

Determining the Link Between Genome Integrity and Seed Quality

Robbie Michael Gillett

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Abstract

Seed quality is of paramount importance to agriculture, food security and plant conservation programs. However, our understanding of the molecular aspects determining seed quality is far from complete, and the influence of environmental conditions during seed development and post-harvest storage are poorly characterised. There is accumulating evidence that DNA damage, response and repair mechanisms are major factors that control germination performance. Here, levels of DNA damage were analysed to determine the effects of unfavourable environments during seed development and deterioration in storage on genome integrity. In response to DNA damage, plants display a highly specific transcriptional response to double strand breaks (DSBs). This response was found to be highly sensitive to seed ageing. A slight reduction in seed vigour accompanied a reduced ability to respond effectively to DNA damage upon imbibition, indicating that an impaired DNA damage response is an early symptom of seed deterioration. Analysis identified the specific forms of DNA damage associated with seed ageing. Levels of single strand DNA breaks (SSBs) increased with loss of seed viability. Similarly DNA base damage, in the form of 8oxoG residues, increased in the dry seed following accelerated ageing. Evidence of repair of this base damage was identified within nine hours imbibition. Suboptimal temperature in the maternal environment did not significantly influence levels of these lesions, consistent with pathways mitigating DNA damage active during seed development. A requirement for antioxidant activity in genome protection was also studied using mutants with reduced levels of the antioxidant vitamin C. Understanding the molecular differences seen between high quality seeds and those that have undergone different degradation conditions provides insight into the process by which seeds lose the capacity to germination. Future analysis in different species will determine the utility of DNA damage related biomarkers for seed quality and identify potential genetic targets to improve seed performance.

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Abbreviations

8-oxoG	7,8-dihydro-8-oxoguanine
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia mutated and rad3 related
B-NHEJ	backup non-homologous end joining
BER	base excision repair
bp	base pairs
Col-0	Columbia 0 (a wild-type ecotype of <i>Arabidopsis thaliana</i>)
CT	control temperature (18°C-22°C; 16 hour day; table 3.1)
DDR	DNA damage response
DMC1	DNA meiotic crecombinase 1
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependant protein kinase
DSB	double strand break
DSBR	double strand break repair
dsDNA	double stranded DNA
dNTP	deoxyribose nucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
FPG	formamidopyrimidine [fapy]-DNA glycosylase
GFP	Green fluorescent protein
GUS	β -glucuronidase
H2AX	histone 2a isoform
HR	homologous recombination

HT	high temperature conditions (25°C-28°C; 16 hour day; table 3.1)
IR	ionising radiation
KU	KU antigen
LB media	Luria Bertani medium
LB	left border
LIG4	DNA ligase 4
LT	low temperature conditions (14°C-16°C; 16 hour days; table 3.1)
MGT	mean germination time
MMC	mitomycin C
MMEJ	microhomology-mediated end joining
MMS	methyl methanosulfonate
MMR	mismatch repair
MRE11	meiotic recombination 11
MRN	MRE11/RAD50/NBS1 complex
MS	Murashige and Skoog basal medium
NBS1	Nijmegen breakage syndrome
NER	nucleotide excision repair
NHEJ	nucleotide excision repair
nm	nanometers
PARP	poly-ADP-ribose polymerase
PCD	programmed cell death
PCR	polymerase chain reaction
PIKK	phosphatidylinositol 3-kinase-related kinase
RAD	radiation sensitive

RH	relative humidity
ROS	reactive oxygen species
RNA	ribonucleic acid
RNR	ribonucleotide reductase
SMR	siamese-related
SSB	single strand break
ssDNA	single stranded DNA
T-DNA	transfer DNA
TE	10 mM Tris-Cl 1 mM EDTA pH 8.0
TK	thymidine kinase
TSO2	TSO (meaning 'ugly' in Chinese) 2
UV	ultraviolet
WT	wild-type
WEE1	WEE1 G2 checkpoint kinase

1. Introduction

1.1 DNA damage in plants

Plants are continuously exposed to environmental stresses and oxidative species generated by cellular metabolism which compromise genome integrity throughout their developmental cycle (Waterworth et al., 2011). Unrepaired DNA damage can lead to arrested growth and development, mutations and cell death. Organisms have therefore evolved complex networks of DNA repair and response factors to restore genome integrity (Lindahl and Wood, 1999). Due to an absolute dependence of plant growth on cell division in the root and shoot apical meristems, the potentially harmful effects of DNA damage in these regions must be particularly tightly controlled (Heyman et al., 2014). Any mutations arising in meristematic cells have the potential for transmission to the next generation (Ries et al., 2000). Plants are sessile, photosynthetic organisms, which renders them particularly vulnerable to DNA damage through a dependence on light for photosynthesis, and consequently constant UV exposure, and the inability to escape genotoxic stresses in their environment. Furthermore, seeds are exposed to high levels of genotoxic stress as a consequence of desiccation/rehydration cycles and extended periods in a dry quiescent state, which are associated with high levels of oxidative damage (Bailly, 2004; Rajjou et al., 2012; Waterworth et al., 2015).

1.1.1 Ultraviolet damage

An absolute reliance on sunlight for photosynthesis exposes plant cells to ultraviolet (UV) radiation. UV light lies between 10nm and 400nm on the electromagnetic spectrum. UV-A (320nm-400nm) and UV-B (280nm-320nm) are the only forms capable of penetrating the Earth's atmosphere, with around 95% being UV-A. UV-B however is responsible for most UV-induced DNA malformations in plants (Jansen et al., 1998), typically accumulating as dimerization of adjacent pyrimidine bases. There are two main UV-induced lesions; cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PPs). CPDs constitute around 75% of these lesions (Waterworth et al., 2002), and if left unrepaired these dimers are capable of blocking transcription and DNA replication. The removal of pyrimidine dimers occurs through either of two pathways, the light-dependant direct reversal

pathway or 'dark repair mechanisms' of nucleotide excision repair (Waterworth et al., 2002).

1.1.2 Alkylation and hydrolysis

Alkylation occurs when hydrocarbons are bound to nucleotide residues. Although DNA damage can occur through alkylation, the process also includes methylation events which generate epigenetic changes within genomes. The methylation of cytosine at the 5-carbon location typically causes transcriptional silencing and is used to commonly "switch off" genes (Gehring and Henikoff, 2008). Despite the utility of alkylation in epigenetics, it can also cause genotoxicity in cells. Induction of alkylation using ethylmethane sulfonate (EMS) in *Arabidopsis* has been used to generate point mutations allowing for forward genetic screening of biological processes. EMS induces methylation in guanine which promotes guanine-thymine base pairing and C to T transitions (Qu and Qin, 2014).

1.1.3 Oxidative damage

Reactive oxygen species (ROS) are generated as part of normal plant cellular metabolism. In excess, ROS can be extremely destructive within a cell, causing damage to macromolecules including lipids, proteins, RNA and DNA. In animals oxidative lesions are up to 15 times more abundant than depurination, the next largest contributor to base damage (Bray and West, 2005).

Despite their destructive nature, ROS are also utilised within plant cells as signalling molecules in processes such as PCD, pathogenic responses and responses to stress (Mittler, 2002). The levels of ROS rise under stressful conditions, threatening the oxidative equilibrium and reaching an upper threshold at which damage begins to occur. As plants are sessile organisms they must tolerate exogenous stresses in their environment such as heavy metals, heat and water availability which can generate additional oxidative damage (Sethy and Ghosh, 2013; Wen et al., 2016). Oxidative lesions to DNA can cause mutations which, if left unchecked, could result in permanent mutations or cell death.

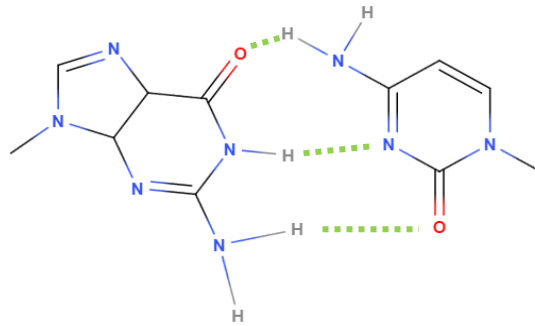
High levels of oxidative damage associated with desiccation/rehydration cycles and the inactivation of protection and repair mechanisms in the desiccated state repair is considered a major cause in reduction of seed quality (Bailly, 2004; Rajjou et al., 2012; Sano et al., 2015). The very low metabolic activity in the dry seed means repair cannot occur until imbibition and the commencement of germination processes.

One of the main genomic lesions associated with oxidative damage is 8-oxo-7,8-dihydroguanine (8-oxoG), a modification of the nucleotide guanine (Kanvah et al., 2010). Guanine is the most commonly oxidised base due to its low oxidation potential, readily causing reactions with ROS, especially when as part of a chain of successive guanine molecules (GG..) (Saito et al., 1998). Increased presence of 8-oxoG has the potential to cause mutagenesis due to erroneous pairing with adenosine (rather than cytosine) causing C-to-A transitions and subsequently G-to-T transitions following excision of the 8-oxoG (Shibutani et al., 1991) (Figure 1.1). These transitions are incorporated during DNA replication and mutations are passed on to daughter cells.

Several studies now identify that damage to nuclear DNA is a major factor in the reduction of seed quality, especially during extended periods of storage (Balestrazzi et al., 2011; Waterworth et al., 2016). Orthodox seeds have enhanced mechanisms to handle the generation of excessive reactive species in order to survive storage whilst minimising damage. By lowering seed moisture content and thereby reducing metabolic activity, production of ROS is reduced (Rajjou et al., 2012). However due to the cessation of most metabolic activities any enzymatic repair activity is minimal. ROS build-up in tissues, and resulting damage, is therefore irreparable until resumption of normal metabolism (Smirnov, 1993). Upon seed imbibition, any ROS accumulated within cellular structures are released, causing a large 'burst' of oxidative damage which must be countered by detoxifying enzyme and antioxidant systems, along with the repair of any damage they cause (Bailly, 2004). The systems utilised to handle this burst of oxidative damage can easily be overwhelmed, especially in lower quality seeds which show a reduction in antioxidant activity, subsequently lowering seed vigour and final germination performance (Pukacka, 1991; De Vos et al., 1994; Bailly, 2004). Excessive

storage over long periods of time, or in high temperature and humidity environments, drastically increase ROS production in planta, leading to an increase in damage and reduction in overall seed quality (Bailly, 2004; Tian et al., 2008).

[A]



[B]

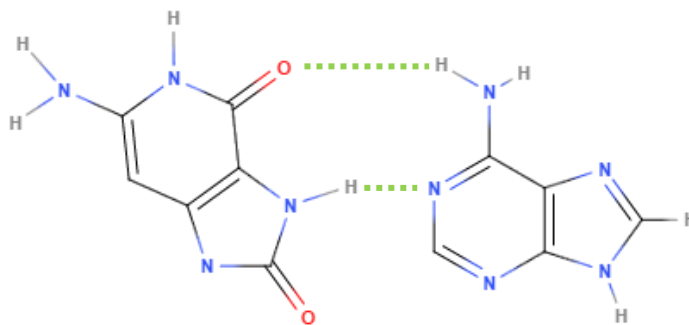


Figure 1.1 Guanine:cytosine base pairing

Guanine typically pairs to cytosine in the genome through three hydrogen bonds [A]. Following oxidation of the C8 molecule guanine changes conformation and binds to adenine to create a Hoogsten base pair [B].

Oxidation to DNA can lead to mutations through transversions and mis-pairing but can also cause single strand breaks through normal repair processes. Oxidised guanine molecules are repaired via the base excision repair (BER) pathway which can generate single strand breaks in order to remove the oxidised base and replace it with a newly synthesised guanine molecule (Section 1.2.4). Strand breaks are highly genotoxic as they increase the risk of losing genetic information if left unrepaired.

1.1.4 DNA strand breaks

Single and double strand DNA breaks threaten genomic stability due to the potential for loss of genetic information. Excess amounts of reactive oxygen species are responsible for the majority of DNA strand breaks through oxidation of the phosphodiester bond between nucleotides (Britt, 1996). The induction of strand breaks is possible through several methods including exposure to gamma radiation and introduction of radiomimetic compounds, most of which break the phosphodiester bonds between nucleotides on the same strand through increased levels of ROS in the cell.

Single strand DNA breaks (SSBs) typically pose less of a cytotoxic threat than DNA double strand breaks (DSBs) as the former retains the availability of the intact strand to replace missing nucleotides via complementary base pairing. SSBs are also often the intermediate by-product formed in DNA repair processes. Following damage to a base, the repair machinery will usually generate SSBs by excising the damaged base or a short stretch of nucleotides and replacing it with newly synthesised DNA (Bray and West, 2005). A single strand break can be created in different ways, including by a dual AP lyase/glycosylase enzyme that recognises certain base lesions and remove the affected nucleotide, leaving an abasic site (such as FPG and oxidised guanine molecules).

Double strand DNA breaks are the most cytotoxic form of DNA damage, with a single unrepaired DSB being sufficient to cause death in yeast cells (Bennett et al., 1996). This is due to the potential loss of genetic information on both strands of the DNA and the loss in the continuity of the double helix. If left unrepaired, double strand breaks can cause fragmentation of chromosomes,

with substantial loss of genetic information following DNA replication and cell division. Depending on the level of DSBs or the amount of genetic information lost, this can result in cell death or severe mutations. DSBs can be induced through ionising radiation, where both strands of the duplex are subjected to simultaneous oxidation causing breaks to form, or through compounds such as bleomycin. Bleomycin is thought to primarily act by chelating metal ions, which in turn enhance the production of superoxide radicals following interaction with oxygen. These localised high concentrations of ROS attack the phosphodiester bonds resulting in the detachment of neighbouring nucleotides on both DNA strands. Double strand breaks need to be repaired quickly in order to avoid cellular catastrophe and there are two highly conserved mechanisms that deal with repair of these lesions, homologous recombination (HR) and non-homologous end joining (NHEJ).

1.2 DNA repair in plants

Accumulation of damage to the genome is highly undesirable because of the potential consequences for plant growth and development, but also the contribution of mutations to the germline and ultimately to the next generation. DNA damage must either be prevented or repaired in order to retain the original, intact genome. Thus, due to a highly stressful life cycle, DNA repair mechanisms need to be efficient, especially given that plant meristem cells give rise to reproductive tissues at a relative late stage of development (Sablowski, 2004). However, plants have developed particularly robust mechanisms to both protect themselves from genome damage and to repair any lesions that do occur (Waterworth et al., 2011). The mechanisms behind DNA repair are broadly conserved across eukaryotes, reflecting their fundamental importance for cellular survival. However, there are several key differences between eukaryotes. For example, plants, unlike mammals, do not contain p53 or Chk1 important mediators of cell cycle progression during genotoxic stress. Although our understanding is more advanced in animal and yeast models, our knowledge of repair mechanisms in higher plants has greatly increased in the last two decades. In particular dependence of plant genome modification technologies on DSB repair pathways, also termed DNA recombination pathway, has focussed research on the underlying

mechanisms. Plants appear to have higher tolerance for DNA damage in comparison to other organisms, possibly because they are exposed to higher levels of genotoxic stress as a consequence of their sessile, auxotrophic nature (Killion and Constantin, 1971; Britt, 1996).

1.2.1 DNA repair synthesis

In all forms of repair in plants, with the exception of direct reversal mechanisms that operate on alkylated nucleotides and UV dimers, synthesis of new DNA is required to replace the damaged or excised nucleotides. Deoxyribonucleotides (dNTPs) are synthesised by *de novo* or through salvage pathways and are used by DNA polymerases in repair synthesis (Bray et al., 2008).

Ribonucleotide reductase (RNR) is required during *de novo* synthesis of dNTPs. RNR is an essential enzyme which acts in a rate limiting step to catalyse the reduction of ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs). The protein consists of four molecules comprising two large (R1) and two small (R2) subunits. The R1 units are responsible for binding NDPs and provide feedback on cellular levels to ensure that a constant supply is available for DNA repair and RNA synthesis. The larger R2 subunit is responsible for the production of dNDPs from NDPs via an intrinsic di-iron tyrosyl radical cofactor (Kolberg et al., 2004). In *Arabidopsis* three genes encoding R2 subunits have been discovered: *RNR2A*, *RNR2B* and *TSO2* (Wang and Liu, 2006). *TSO2* increases in expression in the presence of double stranded DNA damage induced by bleomycin treatment, and levels rise further in *atr* mutants, and form part of the ATM-mediated DNA damage response (section 1.6.2) (Roa et al., 2009). *RNR2A* and *RNR2B* expression levels rise in the presence of hydroxyurea, a replication-blocking agent. This transcriptional induction is reduced in *atr* mutants, and this induction may play a role in the response to replication stress (Roa et al., 2009). RNR activity and nucleotide production is intrinsically linked to DNA repair, with defective RNR activity in yeast and mammals leading to cell cycle arrest and retarded growth (Kolberg et al., 2004). Levels of dNTPs also rise up to 8-fold following DNA damage suggesting increased levels precede repair synthesis (Chabes et al., 2003; Kolberg et al., 2004). However,

steady state cellular levels of dNDPs are tightly controlled in normal conditions with increased RNR activity leading to increased DNA repair, but also higher mutation rates (Chabes et al., 2003).

The second pathway involved in deoxyribonucleotide synthesis involves the salvaging of deoxyribonucleosides by phosphorylation, generating 5'-monophosphate deoxyribonucleosides. The process is mediated by deoxyribonucleoside kinases (dNKs), which are well characterised in mammals but are absent in fungi (Sandrini and Piškur, 2005). The salvage mechanism and the specific enzymes required vary widely throughout kingdoms, with fruit fly (*Drosophila melanogaster*) containing one gene encoding four different dNK activities, whereas humans have four different dNK genes for the same activity. The four genes involved in the human salvage pathway are thymidine kinase 1 (TK1), thymidine kinase 2 (TK2) deoxycytosine kinase (dCK) and deoxyguanosine kinase (dGK) (Welin et al., 2004). In *Arabidopsis* two genes have been identified with sequence similarity to the human TK1 gene and have been termed *TK1a* and *TK1b*. One further gene has shown dGK, dCK and deoxyadenosine kinase (dAK) properties, suggesting three genes are involved in the salvaging pathway in *Arabidopsis* (Clausen et al., 2012). Originally *TK1a* and *TK1b* were thought to have analogous roles in the salvaging of deoxyribonucleosides. This was due to single *tka1* or *tka2* knockout plants showing no phenotypic differences but double *tka1/tka2* mutants dying at an early stage; this also identifies the importance of scavenging early in plant development (Clausen et al., 2012). However recent studies show transcriptional induction of *TK1a* transcripts, but not *TK1b*, following UV treatment. This suggests that *TK1a* is the major gene involved in salvaging as a response to DNA damage (Pedroza-García et al., 2015). Upregulation of *TK1a* also led to increased resistance to genotoxic lesions including base lesions and double strand breaks, suggesting a role for TK1a in several DNA repair pathways (Pedroza-García et al., 2015).

1.2.2 Single strand DNA damage and base lesions

DNA damage lesions incurred by a single strand of the DNA duplex represent a less genotoxic threat to genome stability than DSBs. Single stranded DNA damage vary in abundance and severity, with photoproducts being repaired

without the need for breaking the strand in a “direct reversal” reaction, whereas nucleotide excision repair requires excision and replacement of oligonucleotides from the genome (Wood, 1996).

1.2.2.1 Photoreactivation and direct reversal

DNA is capable of repairing ultraviolet (UV) photoproducts through direct chemical reversal which does not require the incision of the phosphodiester backbone of DNA. Two enzymes are required for the reversal of the two UV-B induced photoproducts in *Arabidopsis* in light-dependent reactions driven by UV-A and blue light (Sancar, 1996). Cyclobutane pyrimidine dimers (CPDs) are removed using CPD photolyase and pyrimidine (6-4)pyrimidones (6-4PPs) are removed with 6-44PP photolyase. Plants deficient in either of these photolyases display increased UV sensitivity (Sancar, 1994; Jiang et al., 1997; Waterworth et al., 2002). Due to the lack of photosynthetic activity in seeds and protection from UV light by the seed coat, photolyases are absent in *Arabidopsis* seeds but are found in large amounts in emerged seedlings (Pang and Hays, 1991).

1.2.2.2 Base excision repair

Base damage is repaired by base excision repair (BER), a very well-conserved pathway which is found throughout all kingdoms with examples of the mechanism present in bacteria through to humans. BER is mainly responsible for the repair of smaller lesions such as oxidative damage to bases, deamination, removal of methylation and the removal and replacement of incorrect bases (Córdoba-Cañero et al., 2009). The BER pathway is of great importance due to the prevalence of large levels of base damage in comparison to other damage types. The highly conserved nature of the pathway reflects the necessity for cells to cope with large amounts of base lesions that occur in several variations. Each base lesion that occurs in genomic DNA has a specific glycosylase activity associated with removal followed by strand incision in order to remove the damaged bases, again with the exception of direct reversal reactions. These glycosylases generate single strand breaks as an intermediate product of repair which in dry *Zea mays* seeds accounted for 38 AP sites per 10^6 bases (Dandoy et al., 1987).

Uracil is erroneously incorporated into DNA or produced through hydrolytic deamination of cytosine at estimated rates of 100 lesions per cell per day in a genome of 3,000 Mbp (Britt, 1996). The importance of this pathway in seeds is also exemplified through the increased accumulation of 8-oxoG lesions through storage and imbibition (Chen et al., 2012). 8-oxoG is the main oxidative lesion in plants and repair occurs through the BER pathway (Kimura and Sakaguchi, 2006).

Although different organisms have distinct BER pathways, the steps involved follow a similar pattern and begin with the recognition of a base lesion by lesion-specific DNA glycosylases. In the case of oxidation and the 8-oxoG lesion, the glycosylase used is either FPG or OGG, with plants the only organisms known to contain both (Murphy and George, 2005). These glycosylases cleave the damaged base, creating an apurinic or apyrimidinic site, before an AP endonuclease or AP lyase incises the phosphate backbone 5' to the abasic site (Britt, 1996). This leaves the strand with two exposed ends, a 3'-OH and a 5'-deoxyribose-5-phosphate (5'dRP). This break is then filled with the correct nucleotide (short-patch BER) or there is resection along the strand and 2-10 nucleotides are replaced (long-patch BER) (Córdoba-Cañero et al., 2009). The gap is filled by a DNA polymerase and a phosphodiester bond is generated between the 3'-OH and the 5'-phosphate using a DNA ligase, thought to be DNA ligase I (Córdoba-Cañero et al., 2011).

1.2.2.3 Nucleotide excision repair

Nucleotide excision repair (NER) is responsible for the removal of a wide variety of lesions in genomic DNA, ranging from photoproducts that are unrepaired by direct reversal through to bulky helical distortions (Kunz et al., 2005). NER is extensively studied in mammals due to the contribution of the repair system in photosensitive syndromes such as xeroderma pigmentosum (XP) (Friedberg, 2001).

The multistep mechanism of NER relies on detection of lesions, incision of the strand either side of the damaged nucleotides, excision of several nucleotides surrounding the lesion and synthesis/ligation of new DNA to fill the gap. There are two pathways involved in the detection of damage in the NER pathway.

One is closely coupled to transcription, directly interacting with the transcription complex, and is named the transcription coupled repair pathway (NER-TCR). This pathway preferentially targets genomic regions that are undergoing transcription in order to avoid the transcriptional machinery encountering deleterious lesions (Kunz et al., 2005). The alternative pathway is known as global genome repair (NER-GGR) and is responsible for genome-wide repair through recognition of UV-products by DNA Damage Binding proteins 1 and 2 (DDB1/DDB2) (Scrima et al., 2008).

Following recognition both pathways rely on DNA unwinding at the site of damage, mediated by general transcription factor-IIH (GTFIIH). In humans the NER pathways is well studied and excision relies on either XERODERMA PIGMENTODUM GROUP B (XPB) or a complex consisting of two proteins: XPD and EXCISION REPAIR CROSS-COMPLEMENTING PROTEIN 1 (ERCC1), with homologues present in *Arabidopsis* (Liu et al., 2000; Vonarx et al., 2006). After the affected lesion, along with several nucleotides either side, are removed, DNA polymerase is involved in synthesis of nucleotides to replace the removed oligonucleotide. Ligation occurs, restoring the affected strand to its original sequence.

1.2.3 Repair of double stranded DNA breaks

Double stranded, or chromosomal, breaks (DSBs) are the most cytotoxic form of DNA damage within the genome. The cellular consequences of these deleterious lesions increase in severity in actively dividing cells, such as the plant meristems, where cell division in the presence of DSBs produces clonal mutant cells (Sablowski, 2007; Waterworth et al., 2015). As such the repair of double strand DNA breaks is imperative in maintenance of genomic stability. The persistence of these lesions upon germination, when plant meristems are activated, poses a serious threat to subsequent cell lineages arising from the undifferentiated stem cells (Waterworth et al., 2016). There are two major repair mechanisms for these lesions. Homologous recombination (HR) utilises a nearby sister strand in order to direct base-pairing-related repair, and error-prone non-homologous end joining (NHEJ), or illegitimate recombination, which does not require a template and randomly joins broken DNA ends.

1.2.3.1 Homologous recombination

Homologous recombination (HR) relies on the availability of a highly similar sequence in the genome to guide repair activity. Initially, following the detection of a DSB, exonucleases resect DNA creating long 3' single strands. From this point there are three separate pathways to DSB repair; single strand annealing (SSA), synthesis dependant strand annealing (SDSA) and the double strand break repair model (DSBR) (Figure 2.2).

SDSA and DSBR are initiated in a similar fashion: double strand breaks are bound by stabilising proteins, notably the MRN (MRE11, RAD50 and NBS1) complex, followed by 3' resection, generating long single stranded regions of DNA (Jazayeri et al., 2006). These strands invade DNA duplexes in the search for homology mediated by RAD51. RAD51 is essential in homologous recombination and indispensable for meiotic development of gametes. Despite this, *rad51* mutant *Arabidopsis* plants are otherwise phenotypically normal, even in the presence of DSB-inducing toxins, suggesting minor roles for HR in maintenance of plant genomic integrity in vegetative tissues (Li et al., 2004). Following the RAD51-mediated invasion of the resected strand into duplex DNA, one strand of the invaded duplex is displaced, creating a 'D-loop' (Waterworth et al., 2011). This is the point at which SDSA and DSBR repair mechanisms diverge.

In the SDSA pathway the invading strand uses homology from the template duplex DNA to restore the original sequence. The invading strand then dissociates from the invaded duplex and re-joins with the free DNA end at the other side of the break. Any missing bases are synthesised based on the newly synthesised DNA, usually restoring the original sequence. If the invading strand does not dissociate and nucleotide synthesis continues until the end of the invaded chromosome, this is termed break-induced repair (BIR). However, BIR is not considered a major contributor to DSB repair in plants (Schubert et al., 2011).

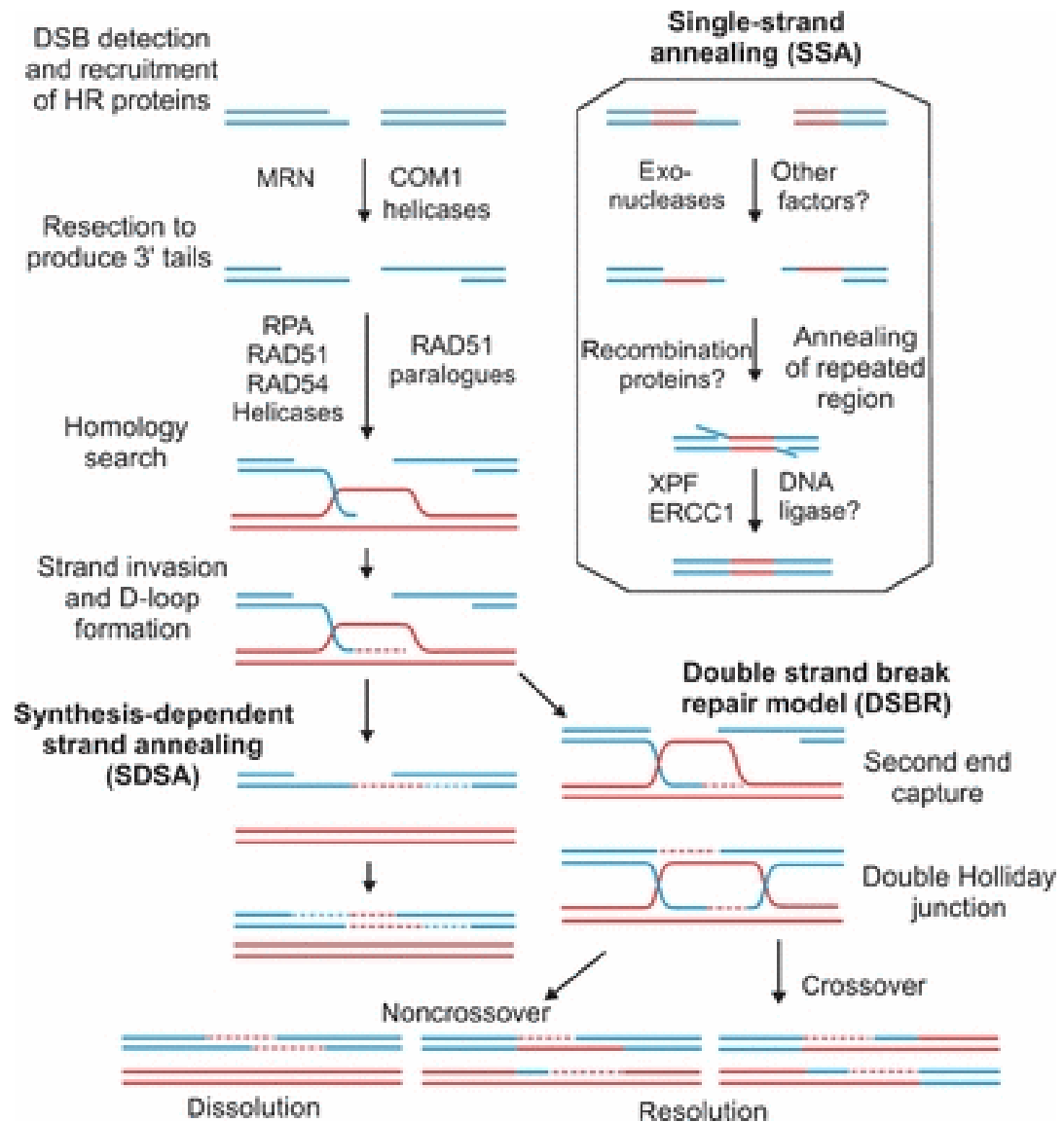


Figure 1.2 Homologous recombination pathways

The three different pathways involved in HR. Single strand annealing (SSA) is the most common pathway and repairs most double strand breaks occurring between repeated sequences, however the intervening sequence is lost upon ligation. Synthesis dependant strand annealing (SDSA) best described HR products in plants and the double strand break repair model (DSBR) shows how chromosomes have the capacity to cross-over, a feature utilised during meiosis. (Waterworth et al., 2011)

DSBR is capable of generating crossovers between the damaged chromatid and the template chromatid. Following D-loop formation, the displaced strand joins to the 3' site on the opposite strand of the original break. This causes the two chromatids to become linked through homology base pairing and a double Holliday junction is formed. Resolution of Holliday junctions results in one of two final products. In one, the invading strand will "crossover" to the duplex DNA in which it was searching for homology and become permanently associated with that chromatid from the break site to the 5' end. The second resolution exchanges the region of homology where the invading strand enters the duplex DNA with the original displaced strand. This replaces the intervening sequence between the break site and the end of the 3'resection. The final resolution is based on where the intervening DNA strand and the dissociated strand are cut (Mazon et al., 2010).

Single strand annealing (SSA) is thought to be the most utilised method of efficient HR repair in plants and occurs when DSBs form in stretches of DNA containing sequence repeats (Siebert and Puchta, 2002). Unlike other HR pathways, SSA does not require a template sequence, rather following resection of 5' ends the two single-stranded, repeating regions ligate together and the intervening sequence is lost.

1.2.3.2 Non-homologous end joining

The second mechanism of double strand break repair (DSBR) is error-prone non-homologous end joining (NHEJ), which repairs DSBs independently of DNA sequence. NHEJ is the predominating mechanism of DSBR in organisms with larger genomes, such as higher plants and animals, unlike yeast and the moss *Physcomitrella patens* in which HR is the principal mechanism. Most DSBR in somatic tissues is performed by NHEJ pathway(s) in most eukaryotes, and there is an incredible flexibility in the forms of DNA broken ends this mechanism is capable of repairing. In the canonical (classic) NHEJ pathway (c-NHEJ), which is conserved across eukaryotes, Ku70 and Ku80 proteins initiate repair by binding to either end of the broken duplex DNA. This is followed by recruitment of the MRN (Mre11-Rad50-Nbs1) complex to the site of broken DNA ends, where end-processing begins to allow the XRCC4-DNA ligase 4 (Lig4) complex to initiate joining of the broken ends (West et al.,

2000; West et al., 2004). Because NHEJ is reliant on processing of broken DNA ends and ligation of the final product, with no homology necessary, it is often associated with varied levels of deletions and insertions (Roy, 2014).

Higher plant NHEJ mutants still retain capacity to repair double strand breaks, although their exact roles and contribution to the overall processing of double strand breaks is yet to be fully elucidated (Bray and West, 2005). Unlike in mammals, in which their counterparts are often lethal, mutants such as *ku70/ku80* and *lig4*, are phenotypically indistinguishable from wild type plants under physiological growth conditions. These mutants do display sensitive to DSB-inducing treatments such as X-rays, indicating that they have a major role to play in enhanced genotoxic stress (Riha et al., 2002; West et al., 2004). *ku70* and *ku80* mutants also show telomere length abnormalities, indicating a function in telomere maintenance (West et al., 2002; van Attikum et al., 2003).

1.3 Responses to DNA damage in plants

Upon detection of DNA damage a cascade of signalling and repair proteins are activated to quickly and efficiently ensure that genome integrity is not compromised. The DNA Damage Response (DDR) is a well conserved pathway that initiates in the presence of double strand DNA breaks and is mediated by the ATAXIA TELANGIECTASIA MUTATED (ATM) protein.

1.3.1 ATM and ATR: the signalling kinases

The initial responses are coordinated by two phosphoinositide-3-kinase-related protein kinases (PIKKs) in plants. ATAXIA TELANGIECTASIA MUTATED (ATM) mainly co-ordinates the response to double strand break and ATAXIA TELANGIECTASIA MUTATED and Rad3 related (ATR) typically responds to replication defects. These two PIKKs activate several downstream responses including cell cycle arrest, transcriptional induction of DNA repair associated proteins, protein phosphorylation, cell death and further activation of other signalling and repair molecules (Culligan et al., 2006; Waterworth et al., 2016) Garcia 2003.

ATM and ATR play central roles in mediating the DNA damage response (DDR) in plant. In response to detection of damage they activate phosphorylation signalling cascades which trigger DNA repair factors, cell

cycle checkpoints and PCD. *Arabidopsis atm* mutants are hypersensitive to ionising radiation-induced double strand DNA breaks (Garcia et al., 2003). The downstream effects of ATM activation by DSBs in *Arabidopsis* include the upregulation of hundreds of repair related genes (the transcriptional DNA damage response) (Culligan et al., 2006), chromatin remodelling activities and histone modifications, specifically the phosphorylation of histone H2AX, a modification localised to DSB sites (Rogakou et al., 1999). ATR on the other hand acts on single strand defects formed during replication or through further processing of double strand breaks (Ünsal-Kaçmaz et al., 2002; Sancar et al., 2004; Culligan et al., 2006).

1.3.2 Transcriptional changes upon detection of DNA damage

One specific response mediated by the DDR in plants is the transcriptional induction of hundreds of genes in the presence of DNA damage. This is largely dependent on ATM; however ATR is also responsible for minor alterations in transcript abundance (Molinier et al., 2005; Culligan et al., 2006). Transcripts associated with the transcriptional DDR are enriched in genes involved in DNA metabolism, cell cycle control, chromosome structural changes and DNA repair. *RAD51*, encoding a protein involved in homologous recombination, displays 186-fold induction upon γ -irradiation. PARP-2, which is involved in detecting and signalling around DNA single strand breaks, displayed over 130-fold induction of transcript levels. Other transcripts encode proteins with roles in nucleotide synthesis, such as thymidine kinase (TK) and ribonucleotide reductase (RNR), which displayed fold increases in transcript levels following 100Gy irradiation of 46 and 37 respectively.

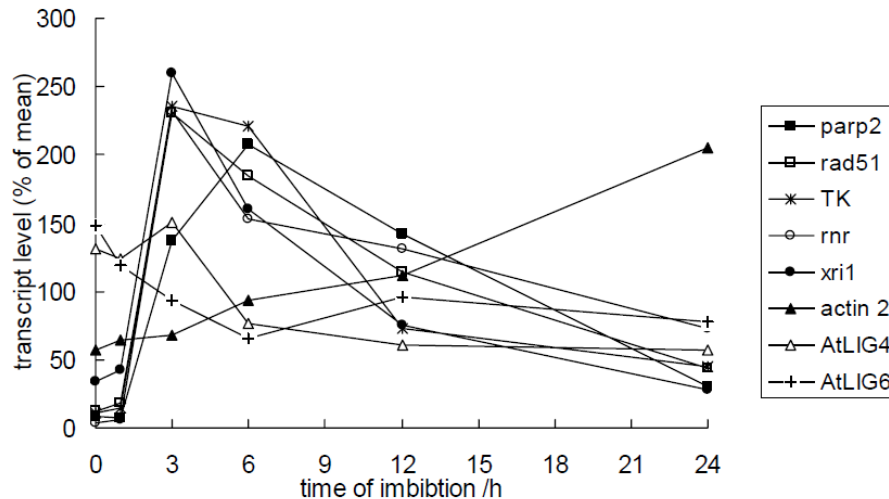


Figure 1.3 Upregulation of repair transcripts following imbibition

The activation of the DNA double strand break transcriptional response following imbibition. (Waterworth et al., 2011)

Although a transcriptional response to DNA damage forms part of the DDR in several organisms, the subset of genes induced varies substantially, indicating high divergence between kingdoms. Although NHEJ is the major DSB pathway in higher plants, no significant changes were observed in genes associated with this mechanism immediately following gamma irradiation, in contrast to the increases observed in HR-related genes (Culligan et al., 2006). Changes in *LIG4*, encoding a core enzyme responsible for the ligation stages of NHEJ, exhibited an ATM-dependent increase in transcript levels; however this responses occurred several hours after irradiation (Garcia et al., 2003). The lack of transcriptional induction of NHEJ is thought to be attributable to constitutive NHEJ activity pathway in cells of higher plants, but also that post-translational control by phosphorylation is likely to be the major mechanism which regulates DDR associated factors in plants, as in other eukaryotes (Matsuoka et al., 2007).

1.3.3 Cell cycle control

The ability to control progression of the cell cycle is important in the presence of DNA damage to extend time for repair and minimise the perpetuation of

mutations in the genome. Thus, control of the cell cycle is an integral component of the DDR (Sancar et al., 2004). The regulation of the cell cycle is mainly based on proteins involved in “checkpoints” between different stages of the cell cycle. By stopping a cell with mutations from entering S-stage of the cell cycle, when DNA replication occurs, the mutation will not be passed on to daughter cells, a process particularly important in regions with high cell division (Sancar et al., 2004). Controlling progression through the cell cycle is mainly controlled by cyclin and cyclin dependent kinases (CDKs).

The transition between the resting phase G_1 and S is controlled in *Arabidopsis* by CDKA;1, one of many CDKs in plants. CDKA;1 is highly homologous to Cdc2⁺ and Cdk1 in yeast and animals, illustrating the large amount of conservation in cell cycle control across kingdoms (Zhao et al., 2012). Further regulation is displayed through inhibitory control of CDKA;1 by the WEE1 kinase in response to DNA replication stress in plants. WEE1 acts to phosphorylate two tyrosine residues on CDKA;1, activating cell cycle arrest seen in *Arabidopsis* and maize (*Zea mays*) (Sun et al., 1999; Sorrell et al., 2002). The impact WEE1 has on the cell cycle is exemplified by knockout *wee1 Arabidopsis* lines, which show cell cycle progression in the presence of DNA damage, whereas *WEE1* overexpressing lines display constituent cell cycle checkpoint activation (Sorrell et al., 2002; De Schutter et al., 2007). The activation of CDKA;1 homologues in mammals is induced by a phosphatase known as CDC25, although no functional homologues have yet been identified in plant species (Dewitte and Murray, 2003).

1.4 Seed Biology

The seed is an embryonic plant enclosed in a protective outer coating known as the testa or seed coat (Bewley and Black, 1994). The seed represents an important step in plant evolution which facilitates survival away from the maternal plant, unlike more ancient plants such as ferns and moss which propagate without forming a seed. Seed development is initiated when pollen (male gamete) fertilises a ripened ovule (female gamete), which is followed by formation of the mature seed on the mother plant before release to propagate the next generation.

Typically seeds contain an outer protective layer known as the seed coat or testa, this provides physical protection to the embryo inside which will go on to establish the full plant. In *Arabidopsis*, along with most flowering plants, there is often the presence of an energy rich endosperm. The cells of the endosperm are triploid and the abundance varies between species, with *Arabidopsis* only having one layer of endosperm tissue and crops such as wheat having abundant endosperm. The initial energy requirements for the embryo during germination are supplied by the endosperm in a species-dependent mixture of starch, oils and proteins. In seeds where the endosperm is not present, such as *Pisum sativum* (pea), energy is stored in the cotyledons other notable examples are members of the *Orchidaceae* family where they require a fungal-symbiotic relationship for energy.

Rapid establishment of a strong seedling is crucially important for high crop yields in agriculture and survival of plant species in ecosystems (Finch-Savage and Basel 2016). Rapid, vigorous germination underpins successful seedling establishment. Once the germination process is initiated, the emerging seedling is highly vulnerable to environmental stresses such as suboptimal temperature, drought, or salinity, weed competition, pathogen infection and pest attack.

1.4.1 Seed development and maturation

Dependent on plant species, up to thousands of seeds can be generated on a single plant. Seed development is initiated after fertilisation, subsequently, embryogenesis and morphogenesis are followed by deposition of storage reserves before seed maturation in which orthodox seeds acquire desiccation tolerance (Bewley, 1997). As orthodox seeds reduce in moisture content the cytoplasm transitions to a glassy state, which helps to increase freezing tolerance and reduces damage accumulated during storage through very low metabolic rates (Buitink and Leprince, 2008). During the period of seed development on the mother plant, they can be subjected to stresses that could affect seed quality and final plant performance potentially years later. The impact of light availability on the maternal plant during lettuce seed development was reported to subsequently result in differences in seed qualities, with long day conditions (8 hours of darkness) producing higher

quality seeds than short day conditions (16 hours of darkness) following storage (Contreras et al., 2008). Other conditions which detrimentally affect seed quality during the maturation stage include drought nutrient deficiency that pushes the plant towards survival over reproduction (Peters, 1982; Fenner, 1991; Wang et al., 2012). Stress during seed maturation could be considered to be as a double edged sword, as some studies suggests it can generate resilience to the stress, whilst others suggest it negatively impact on the seed.

Dormancy is a block to germination which prevents germination under favourable conditions of germination, and is more usually associated with temperate species (Graeber et al., 2012; Wang et al., 2012). Primary dormancy, in which seeds are dormant from the time of seed maturity when shed from the mother plant, is a survival strategy to enable temporal and spatial distribution of germination. Prolonged exposure of a non-dormant seed to unfavourable conditions can induce a state of secondary dormancy. Dormancy cycling between a non-dormant and the dormant state is a mechanism which promotes persistence of seeds in the soil seedbank (Cao et al., 2013).

1.4.2 Seed storage

Seeds can be divided into three categories on the basis of desiccation tolerance: orthodox, recalcitrant and intermediate species (Ellis et al., 1991). The capacity to withstand desiccation tolerance is a key trait in orthodox seeds, in which survival in a dry state prolongs seed longevity and enables seeds to survive environmental extremes of low temperature and freezing. Recalcitrant seeds are intolerant of desiccation, whilst species with characteristics between the two groups are known as 'intermediate' seeds. Orthodox seeds can withstand desiccation, typically as low as 10-15% (Bewley and Black, 1994). The low water content of orthodox seeds effectively extends embryo survival. In extreme cases date palm seeds have remained viable for over a 2000 years following desiccation (Sallon et al., 2008). Seeds of temperate regions are typically orthodox, as are most agricultural species, including cereals and grains. The seeds can then be dry stored for long periods of time without losing germination capacity. Recalcitrant species, such as

cocoa (*Theobroma cacao*) and mango (*Mangifera indica*), are generally limited to tropical regions where severe winters are uncommon and thus germinate readily from the fruit.

However, seeds still deteriorate at a reduced rate in the dry state due to very low levels of metabolism (Bewley and Black, 1994; Bewley, 1997). Decline in seed quality is initially seen as a decrease in rapidity and synchronicity of germination. An increasing delay to germination is also accompanied by an increased frequency of abnormal seedlings in many species and the seed lot eventually demonstrates a loss of viability. The ability of a seedling to germinate rapidly and uniformly under a wide range of environmental conditions is known as seed vigour and is an important factor which underpins crop productivity (Finch-Savage and Bassel, 2015b).

The molecular factors which determine resilience of the seed to deterioration are incompletely understood. However, increasing deterioration of macromolecules and cellular structures has long been known to correlate with extending periods in the desiccated state (Waterworth et al., 2015). This deterioration can be exacerbated by unfavourable conditions storage in high temperatures or high humidity. This deterioration is of prime economic importance as manifests as reduced seed quality, reduced vigour and subsequent yield losses (Powell and Matthews, 1984).

Increasing evidence identifies that the ability to prevent or repair this accumulated damage is a key determinant of seed vigour and viability, and moreover that levels of damage and repair capacity directly determine the ability of a seed to germinate and by extension survive (Sano et al., 2015; Waterworth et al., 2015).

1.4.3 Germination

In the germination process the quiescent embryo switches to a metabolically active state. Germination can be split into three phases (Bewley, 1997). Germination commences in Phase I upon imbibition with the uptake of water and the initiation of metabolic activities. Upon imbibition cells within the seed hydrate, although not uniformly, with the micropylar endosperm and the radicle showing the greatest level of hydration early in germination (Manz et al., 2005).

Rapid rehydration of the desiccated seed may be associated with high levels of oxidative damage to macromolecules and cellular structures in the seed. Upon hydration, resumption of metabolism is accompanied by initiation of DNA repair processes. Major metabolic enzymes are present in the dry seed and further proteins are produced with the resumption of transcription and protein synthesis. Furthermore, transcriptional changes can be seen as early as 3 hours into imbibition of *Arabidopsis* seed, including several genes associated with DNA damage responses (Preston et al., 2009; Waterworth et al., 2010). An overview of the different metabolic changes dependent on stage of imbibition can be seen in Figure 1.4 (Bewley and Black, 1994.)

Phase II of germination represents a time where water uptake is complete and is associated with general changes in seed size. Phase II typically varies in duration between species, also depending on the quality of the seed. In maize (*Zea mays*) this period has been shown to extend proportionally to the amount of deterioration (Matthews and Khajeh-Hosseini, 2007). Following testa rupture phase II is completed and further water uptake dictates the start of stage III when the radical emerges. Germination, *sensu stricto*, is completed upon radicle protrusion through the endosperm and seed coat. There is not an absolute requirement for cell division for the completion of germination in *Arabidopsis*, although expression of cell cycle-related transcripts increases around this period (Barrôco et al., 2005) and cell cycle activation is required for effective germination, as cell cycle inhibition substantially slows completion of germination (Masubelele et al., 2005; Waterworth et al., 2016). Cell division is required for further growth and seedling establishment (classed as post germinative growth). The production of a robust seedling measures the success of germination in establishment of a healthy adult plant (Finch-Savage and Bassel, 2015a).

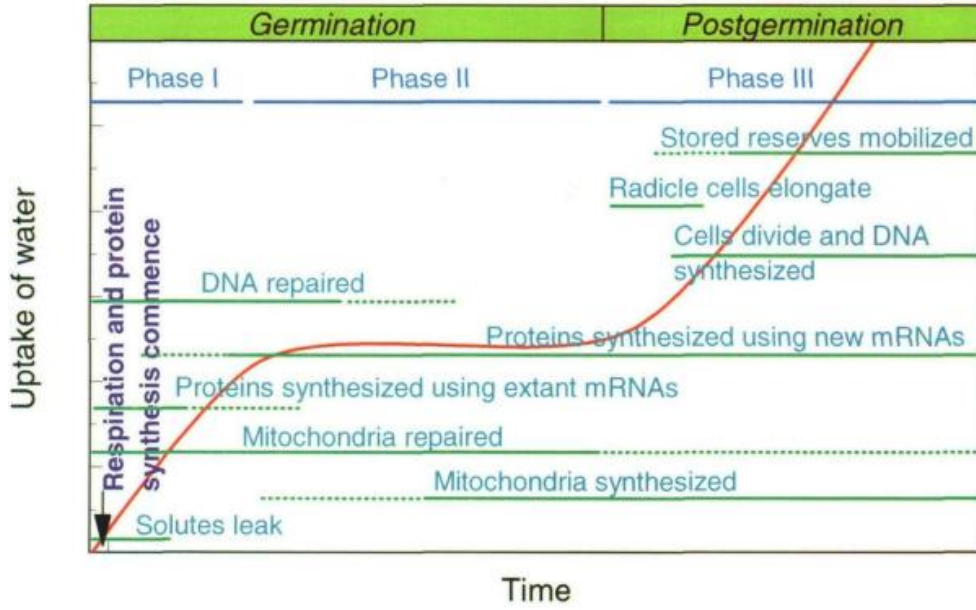


Figure 1.4 Metabolic changes at different stages of imbibition

Events associated with different points during germination. The time taken is heavily dependent on species and germinating conditions, ranging from hours to weeks (Bewley (1997)).

1.4.4 Seed vigour

Seed vigour is a complex trait determined by multiple environmental and genetic factors (Clerkx et al., 2004b). High vigour seeds germinate uniformly and rapidly under a wide range of environmental conditions and are resistant to deterioration in storage. Increasing our fundamental knowledge of seed quality is important because it is a key determinant of final crop yields (Finch-Savage and Bassel, 2015b). Improved resilience of germination to environmental stresses by biotechnological approaches or plant breeding would be an important step in our ability to improve crop yields on marginal land and adapt crop varieties to changing climates (Waterworth et al., 2015). Understanding and predicting seed vigour and viability is also central to conservation of plant germplasm in seedbanks, of both of wild species and cultivars of agriculturally important species. The latter provide a valuable source of genetic resources for crop breeding. However our current understanding of the genetic basis of seed vigour is limited, although multiple factors have been implicated. Seed vigour and seed longevity have been recently reviewed (Rajjou et al., 2012; Finch-Savage and Bassel, 2015b; Sano et al., 2015).

1.4.5 Oxidative damage, repair and seed vigour

Combinations of desiccation/rehydration cycles and quiescence are associated with high levels of oxidative stress (Bailly, 2004; Kranner et al., 2010). Studies over several decades have identified that cellular structures and macromolecules including proteins, RNA, DNA and membranes accumulate increasing damage as the seed deteriorates. Increasing evidence now points to the critical roles of repair and protection mechanisms in maintenance of germination performance. A significant role for tocopherol (vitamin E) in protection against lipid peroxidation in seed longevity and seedling establishment was demonstrated through analysis of mutants deficient in tocopherol synthesis (Sattler et al., 2004).

Oxidative damage to proteins by seed ageing or environment stress has also identified as a key determinant of seed ageing, in as inactivation of key metabolic or repair enzyme activities will impair recovery processes of the

seed. Methionine sulfoxide residues caused by protein oxidation upon ageing are reversed by methionine sulfoxide reductase and levels correlate with longevity in varieties of *Medicago* seeds (Châtelain et al., 2013). Conversion of aspartate to isoaspartyl causes protein misfolding, reversed by L-isoaspartylmethyltransferase1, is associated with ageing in several organisms and is also important determinant of seed longevity (Ogé et al., 2008).

1.4.6 Important roles for genome maintenance pathways in seeds

Studies have established DNA damage repair and response factors as factors very important to seed vigour. Studies by Cheah and Osbourne (1978) identified that seed ageing correlated with loss of genome integrity; rye seeds of 50% viability accumulated SSBs, detected using electrophoretic analysis of genomic DNA under alkaline conditions. Subsequent studies established that DNA repair processes are initiated very early in germination and also that the lag phase in aged seed is accompanied by extended periods of DNA repair associated synthesis (Elder and Osborne, 1993). This supported the idea that a requirement of DNA repair is associated with the increasing delay to germination in aged seed, and that a requirement for repair is limiting for germination (Waterworth et al., 2010).

Studies in several species identified that frequencies of chromosomal abnormalities, including chromosomal fusions, bridges and rearrangements increase in aged seed as deterioration progresses. These represent chromosomal breaks which have been repaired inaccurately by the DSB repair mechanisms in the germinating seed, providing direct evidence that seeds experience high levels of genotoxic stress (Roberts, 1972; Dourado and Roberts, 1984). However, it was concluded that even high quality seeds carry a basal level of chromosomal damage. Desiccation tolerance and quiescence in other organisms has also linked to a remarkable capacity to repair DNA (Waterworth et al., 2011).

Waterworth et al (2010) identified that *Arabidopsis* mutants lacking the NHEJ factors DNA ligase 4 and DNA ligase 6 are hypersensitive to seed ageing. This demonstrated that the ability to repair chromosomal breaks is limiting for seed germination and provided the first genetic evidence of the link between DNA

repair and seed quality, specifically DSBs. Greater sensitivity of the *at4lig/atlig6* double mutants to ageing is consistent with both DNA ligases acting in distinct pathways. Whilst DNA ligase 4 functions in the canonical NHEJ pathway, DNA ligase 6 is unique to plants and is thought to function in alt-NHEJ. Evidence of DSBs was provided by the transcriptional response to DNA damage, which was observed very early in germination of unaged *Arabidopsis* seeds. This response was elevated in mutants lacking DNA ligase 4 and 6, consistent with higher levels of DSBs in these lines. A role for BER in seed longevity was identified by overexpression of the BER enzyme OGG1 in *Arabidopsis* (Chen et al 2012), which removes the oxidised base 8oxoG from DNA. Germination was resistant to accelerated ageing and other germination stresses, indicating the potential of DNA repair factors as genetic targets for crop improvement.

1.4.7 Antioxidant protection

Accumulation of damage in plants is undesirable because of the chance of contributing mutations to the germline or offspring. Several methods have evolved in order to limit damage in seeds, such as condensation of chromatin during seed maturation to limit damage to cellular DNA during desiccation (van Zanten et al., 2011). Large accumulations of reactive oxygen species occur during storage and imbibition, with accumulation of exogenous stresses and endogenous respiration generating enhanced oxidative damage. The removal of excess oxidative damage is reduced by antioxidants such as the non-enzymatic, low molecular weight antioxidants (glutathione and ascorbic acid) and various enzymatic antioxidants (e.g superoxide dismutase). Levels of antioxidants also increase during seed maturation which may help reduce ROS-induced cellular damage at this stage of the plant life cycle (Bailly, 2004)

Ascorbic acid (vitamin C; AsA) is an essential antioxidant in plants, with knock-out mutants not surviving past seedling stage (Dowdle et al., 2007). The major source of production of vitamin C in plants is the L-galactose pathway, although the full understanding of vitamin C synthesis in plants is still not clear with uronic acids thought to contribute small amounts (Wheeler et al., 1998; Smirnov and Wheeler, 2000). Negligible levels of AsA and AsA peroxidase are present in the dry seed but are activated very early in imbibition in response

to increased oxidation pressures and are present prior to seed desiccation during development (Tommasi et al., 1999; De Tullio and Arrigoni, 2003). The importance of vitamin C is clear; however the way it acts in plants is not fully understood. *Trigonella foenum-graecum* seeds soaked in AsA showed greater root length, fresh weight and germination in response to salt-stress (Behairy et al., 2012). Furthermore AsA peroxidase activity has been linked to increased germination in aged *Dasypymm villosum* seeds and increased germination in ascorbate-dependent H₂O₂ scavenging in *Zea mays* (De Gara et al., 2000). However increased AsA was shown to suppress germination in wheat (*Triticum aestivum*) and oxidised AsA has been shown to act as a prooxidant (Ishibashi and Iwaya-Inoue, 2006). Vitamin C therefore has a complex role during the imbibition and germination of seeds that has yet to be fully elucidated.

1.5 Summary and Aims

Plants must overcome several inhibiting factors throughout their life cycle in order to establish and grow to their maximum potential. Although sunlight, water and nutrient availability all affect plant growth and final yield, the success of the mature plant has already been heavily influenced by factors experienced at the seed stage of the plant lifecycle, including seed development on the maternal plant and desiccation during storage.

Our understanding of the molecular factors important to seed vigour is far from complete. Recent studies have established an important role for repair of DSBs in germination and seed longevity and identified that ATM and the DDR, responding to high levels of DSBs in the seed, play important roles in control of the germination process. However, the contribution of other DNA damage lesions and specific repair factors remains to be established. Understanding the molecular basis of seed quality will identify candidate factors for the genetic improvement of crop species and the development of biomarkers for prediction of seed lot quality.

Moreover, seed quality is influenced by environmental conditions experienced during development, quiescence and imbibition. This is particularly pertinent in evaluating the impact of rapidly changing climate conditions on natural plant populations and crop varieties. The study presented here form part of the EU

EcoSeed project, which aims to understand the molecular mechanisms important to seed quality and their relationship with the maternal environment during seed development and storage conditions.

Aims

The overall aim of this project was to determine the influence of the environment during seed development and storage on genome maintenance in the dry and imbibing seed. This was addressed by the following specific objectives:

- **To correlate seed germination performance with levels of specific DNA damage products**

This required the development and application of quantitative methods for analysis of specific forms of DNA damage. In mammalian studies, biomarkers and sensitive methodology to determine levels of genome damage and genotoxic stress are routine, including in clinical use. However, such approaches have not been widely utilised in plants to date. Therefore, optimisation of methodology for the accurate, sensitive and high throughput analyses of DNA lesions is required.

- **To identify the cellular responses to DNA damage in seeds and the activity of these responses in relation to seed quality.**

The DNA damage response is a highly dynamic and specific cellular response to chromosomal damage. Here the aim was to link the activity of these responses to increased levels of genome damage in aged seeds.

- **To determine the relationship between genome damage and the antioxidant ascorbic acid.**

Oxidative damage is considered to be a major source of DNA damage in seed deterioration. One approach to minimise genome damage in seeds, and so enhance seed vigour and longevity, could be to increase protection of the embryo genome through increased levels of seed antioxidant levels.

2. Materials and Methods

2.1 Suppliers

Unless otherwise stated chemicals were obtained from Sigma Aldrich, Poole, UK.

2.2 Equipment

Any centrifugation steps involving 0.5 ml, 1.5 ml or 2 ml microcentrifuge tubes used an Eppendorf minispin microcentrifuge at room temperature at 20238 RCF (relative centrifugal force); equivalent to 14680 rpm (revolutions per minute) unless otherwise stated. Falcon tubes of 15 ml or 30 ml (BD Biosciences) were centrifuged using the 5810R centrifuge with the A4-62 rotor at 3000RPM (1700 RCF) at 20°C unless otherwise stated. 96-well PCR plates (BioRad) were spun briefly in the 5810R centrifuge using an Eppendorf A-2-DWP rotor in the same centrifuge.

PCR reactions were performed in an Eppendorf Mastercycler machine and electrophoresis was carried out in a BioRad mini subcell electrophoresis tank with a basic BioRad power pack. In order to sterilise solutions and equipment a Radwell Herald autoclave was used at temperatures of 121°C for 20 minutes unless otherwise stated. Bacterial densities were determined using an Eppendorf Biophotometer spectrophotometer. Nucleic acid concentrations and purity were determined using a ND-1000 spectrophotometer in the range of 220nm-350nm and analysed with ND-1000 software.

Images of agarose and acrylamide gels were taken inside a SynGene GBOX and quantification carried out using GeneTools software (SynGene). Any other images were taken with a Nikon D90 camera.

2.3 Plant material and mutants

Seed stocks of mutant and wild-type *Arabidopsis* lines were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC: <http://Arabidopsis.info/>) (Scholl et al., 2000) unless they provided by partners of the EcoSeed programme. *Arabidopsis* seeds for use EcoSeed were grown at Warwick University and *Brassica* grown at University Pierre and Marie Curie (section2.5.4). The VTC2 (AT4G26850) T-DNA mutant (*vtc2-5*) had the accession number SAIL_769_H05 (Sessions et al., 2002). The EMS mutation (*vtc2-1*) was donated by the Christine Foyer lab at Leeds University and originally isolated

by Professor Conklin (Conklin et al., 2000). Genotyping was carried out via primer specific (table 2.1) PCR reactions. Primers would amplify with the wild-type allele or T-DNA insertion allele allowing isolation of homozygous mutant lines (section 2.3.1) in 2 week old seedlings grown on ½ MS media. Isolated mutants were then moved to soil to set for seed outlined in section 2.4.

Table 2.1 - List of primers

Primer	Locus	Sequence
actin1_f	AT2G37620	5'-CAATGCCCTGCTATGTATGT-3'
actin1_r		5'-TCACACCATCTCCAGAGTCG-3'
Bo-actin1_f	C5IWW9	5'-AATGGTCAAGGCTGGTTTTG-3'
Bo-actin1_r		5'-GCGTGTGGAAGAGAGAAAACC-3'
actin7_f	AT5G09810	5'-GGTGAGGATATTCAGCCACTTGTCTG-3'
actin7_r		5'-TGTGAGATCCCGACCCGCAAGATC-3'
parp_f	AT4G02390	5'-CCAGATGGGGAAGAGTTGGT-3'
parp_wrt		5'-ACCAGCCCAGTTAGTGAGAC-3'
rad51_f	AT5G20850	5'-GGATCACGGGAGCTCGATAA-3'
rad51_r		5'-TTTCCTCAACGCCAACCTTG-3'
rnr_f	AT3G27060	5'-TCCTAACACCAACCCAGAC -3'
rnr_r		5'-CGCACACAAACTCTCTCTCG -3'
tk_f	AT3G07800	5'-CCTTCGCCGAATCAAGTCAG-3'

tk_r	AT3G07800	5'-TGAAACATGGACCAGAGGCT-3'
silentregion_f		5'-CAATTATTGGTGAAAGGGATTCA-3'
silentregion_r		5'-AGGTTATGACCAATGACACTGC-3'
8oxoGcontaining_f		5'-CAATGCCCCTGC -3'
8oxoGcontaining_r		5'-TCACACCATCTC -3'
VTC2_f	AT4G26850	5'- GGCAAACACAGCAGTCTGAAAC -3'
VTC2_r		5'- TCAGCTTAACGAGGGTCGTCA -3'
vtc2-5_wt		5'- CTTCCGATCTCCTCTTTCTCG -3'
vtc2-5_mut		5'- GAGGCAAGCAGTCAAGAACAC -3'
SAIL_LB1		5'- GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC -3'

A list of primers used for genotyping and RT-PCR. Primer names correspond to the name of the gene for amplification followed by: f – forward primer, r – reverse primer, wt – primer specific for wild-type amplification of gene, tdna – primer specific for amplification when TDNA insertion is present. Primers were synthesised by Sigma aside from 8-oxoG primers provided by DNA technology.

2.3.1 Isolation of double T-DNA insertion knock-out mutants

Two different single T-DNA insertion plants (the F0 generation) were grown on soil before crossing. Plants that had developed 5-6 inflorescences were crossed with plants that had started to form siliques. Mature siliques, open

flowers and white-tipped or matured buds were removed from the first plant. Unopened flower buds were opened using Jeweller's forceps (5Inox, Dumont, Switzerland) and all immature anthers were removed. From the second plant, flowers containing anthers that were shedding pollen were selected and the anther was used to transfer pollen to the stigma of the recipient plant. The pollinated inflorescences were wrapped loosely in cellophane and labelled appropriately and given 15-25 days to form mature siliques. These seeds were then collected and genotyped.

Seeds from the parental lines were grown (section 2.4) and leaves were genotyped using PCR (section 2.7). Plants heterozygous for T-DNA insertions (the F1 generation) were allowed to self-fertilise by segregating these plants from others. Seeds were collected (the F2 generation) and grown under normal conditions (section 2.4). Mendelian inheritance states that one in sixteen of these plants will be homozygous double for the mutation. These plants were identified by PCR genotyping and were allowed to set to seed so a large, renewable stock could be generated to ensure maintenance of the genotype.

2.4 Plant growth conditions

2.4.1 Seed sterilisation and imbibition

Materials

Hyperchlorite solution:

- 10% (v/v) Bleach (5-10% hypochlorite)
- 1% (v/v) Triton-X100

Agar media:

- 0.1% (w/v) Phyto agar (Duchefa Biochemie). Autoclaved and allowed to cool to room temperature

Method

Arabidopsis seeds were suspended in hyperchlorite solution for 10 minutes inside sterile 1.5 ml microcentrifuge tubes. The seeds were then washed 5 times in sdH₂O to remove residual sterilising solution before being added to agar media.

2.4.2 Chlorine gas seed surface sterilisation

Materials

Gas sterilising solution:

- 100 ml Bleach (5-10% hypochlorite)
- 3 ml concentrated HCl

Method

Arabidopsis seeds were placed inside open containers and placed in a sealed desiccator (Nalgene) inside a fume cupboard (pf&f Ltd.). The gas sterilising solution was placed in an Erlenmeyer beaker (Fischer Scientific) inside the desiccator for 3 hours.

2.4.3 Growth of *Arabidopsis*

Materials

MS media (pH 5.7):

- 2.2 g l⁻¹ MS basal medium
- 1% sucrose
- 0.8% Phytoagar (for agar plates)

Method

Seedlings were grown on half concentration Murashige and Skoog (MS) media, plus 1% sucrose, before transferring to soil at up to 2 weeks (Murashige and Skoog, 1962). The media was adjusted to pH 5.7 using KOH. Phyto agar was added and the media was autoclaved before being allowed to cool to 55°C before adding any required antibiotics. The media was then poured into 9cm Petri dishes (Starstedt) and allowed to cool. Sterilised seeds were spread on the MS plates under sterile conditions, sealed with Micropore tape (3 m) and grown in a growth chamber (Sanyo MLR-351) in a 16 hour light period at 70% humidity and 23°C. If the seeds were required to grow past the seedling stage, then they were transferred to soil after 2 weeks.

2.4.4 Plant growth on soil

Materials

SHL growing medium (William Sinclair Horticulture):

- 15% peat 0-5 mm
- 85% peat 0-10 mm
- 204 g m⁻³ N
- 238g m⁻³ P₂O₅
- 408g m⁻³ K₂O (pH6.0)

Method

Plants grown beyond 2 weeks on media were transferred to SHL growing medium. Seedlings were carefully removed from plates and placed into the soil. The plants were then kept in temperature controlled glasshouses at 22°C in a 16 hour/8 hour day/night cycle (commonly referred to as long day conditions).

2.5 Seed production and treatments

2.5.1 Seed production and after-ripening

To ensure seed lots of the different genotypes were comparable, plants were grown at the same time as described in section 2.4. Upon desiccation of siliques, seeds were harvested and stored in non-airtight tubes at ambient temperature and humidity for 2 months to allow adequate after-ripening and removal of primary dormancy (Finch-Savage and Leubner-Metzger, 2006).

2.5.2 Germination assays

Unsterilised seeds were placed on Blue Blotter Germination Paper (referred to as Germination Paper from here onwards) (SGB1924B, <http://www.anchorpaper.com/>) in 90 mm Petri dishes (Sarsdedt) with 7 ml of dH₂O. Seeds on Petri dishes were then stratified for 2 days at 4°C to relieve any dormancy. Following stratification, the Petri dishes were moved to a growth cabinet (Sanyo MLR-351) kept at 16 hour light period at 70% humidity and 23°C. EcoSeed seeds were not stratified and kept under full light conditions (24 hour day) at 15°C in the same Sanyo growth cabinets. Seeds were scored for germination as defined by the protrusion of the radicle from the seed coat (Bewley, 1997). A stereomicroscope (Novex RZB-PL) was used to monitor radicle protrusion at regular intervals. Independent biological replicates were performed with different harvests of both the wild-type seeds and seeds from a mutant background for verification of results. This method

allows for both final germination percentage (viability) and mean germination time (MGT) (vigour; equation 3.1) to be scored.

2.5.3 Accelerated ageing of seeds

To simulate long term storage, high quality, non-dormant seeds were placed inside an open 1.5 ml microcentrifuge tube suspended above a saturated salt solution (100 ml H₂O, 50g KCl) in an air-tight vessel. The seeds were then placed at 35°C producing a relative humidity of 80% for between 1 and 5 days as appropriate. Seeds were scored for viability and vigour loss as described (section 2.5.2) before any further analysis were performed. EcoSeed seeds were generated at Warwick and used different conditions (section 2.5.4).

2.5.4 X-ray treatment of seeds

Seeds were sterilised in 70% ethanol for 5 minutes before being resuspended in sterile H₂O and stratified for 24 hours at 4°C. Ionizing radiation was administered at different doses using a 320 kV X-ray irradiation system (NDT Equipment Services) at a rate of 1 Gy min⁻¹. Seeds were plated individually on ½ MS media in 90 mm Petri dishes and placed upright to allow the roots to grow through the media. Root length was measured daily to determine the effects on root growth using tracking on ImageJ software.

2.5.5 EcoSeed growth and CDT conditions

Arabidopsis seeds grown for the EcoSeed project were produced by the Finch-Savage lab at the University of Warwick under different temperature regimes using Levington F2 compost:sand:perlite 6:1:1. Initially temperature and daylight were kept at 18/22°C for 8h dark and 16h light. Before the first flower appears plants were placed under low temperature, control temperature or high temperature conditions (14°C/16°C, 18°C/22°C, 25°C/28°C). Upon 2/3 brown siliques forming plants were left without water for 7 days in order to complete maturation drying. Seeds were then kept in open bags at 15°C/15% RH until required. *Arabidopsis* seeds that underwent controlled deterioration were kept at 40°C;75% RH for 4 days.

Brassica seeds were grown in John Innes compost at University Pierre and Marie Curie. Seeds were grown at 18°C-22°C in 16 hour day, 8 hour night glasshouses. Seeds were transferred to different maternal temperatures when

seed moisture content reached 75%. Maternal temperatures for these seeds only included a control and high temperature (18°C-22°C, 25°C-31°C). CDT in these seeds was undertaken at Leibniz Institute of Plant Genetics and Crop Plant Research and involved seeds being subjected to 45°C;85% RH for 7.5 days.

2.6 DNA and RNA Extraction from Seed and Leaf Tissue

2.6.1 DNA extraction from leaf and seedling tissue

Materials

Extraction buffer:

- 100 mM Tris-HCl (pH 9.0)
- 200 mM LiCl
- 50 mM EDTA
- 1% SDS

10x T.E. buffer

- 100 mM Tris-HCl (pH 8.0)
- 10 mM EDTA (pH 8.0)

Isopropanol

Method

Leaf or seedling tissues were placed in 1.5 ml microcentrifuge tubes containing 500 µl of extraction buffer then ground using an electric stirrer (Stuart Scientific SS10). The tubes were then centrifuged at 13 000 rpm for 15 minutes at room temperature. 350 µl of the extraction buffer was taken and placed into a fresh 1.5 ml microcentrifuge tube containing the same volume of isopropanol. The tubes were mixed by gentle inversion before being centrifuged for 10 minutes at 13000rpm. The supernatant was removed and the remaining pellet was left to air dry before resuspension in 100 µl T.E. buffer.

2.6.2 DNA extraction from seed tissue

Materials

CTAB buffer:

- 2% (w/v) Cetyl trimethylammonium bromide (CTAB)
- 2 M LiCl
- 50 mM EDTA
- 100 mM Tris-HCl (pH 9.4)
- 1% (w/v) Polyvinylpyrrolidone
- 0.2% (v/v) β -mercaptoethanol
 - 1.4 M NaCl – Used for high quality seed DNA extraction (see section 4.2.1)

Chloroform: isoamyl alcohol (24:1)

Isopropanol

70% ethanol

Resuspension buffer

- 100 mM Tris-HCl (pH 8.0)
- 250 mM NaCl (pH 8.0)
- 20 mM EDTA
- RNase A (10 mg ml⁻¹)

T.E. buffer

- 10 mM Tris-HCl (pH 8.0)
- 1 mM EDTA (pH 8.0)

Method

Seed material (variable quantities, see table 4.1) was ground using a pestle and mortar into a fine powder. The powder was then transferred to a 15 ml falcon tube containing 6 ml of CTAB buffer pre-heated at 60°C. The tube contents were mixed and either incubated at 60°C or, in the case of *Arabidopsis*, not incubated, to aid in the segregation of mucilage from nucleic acid. An equal amount of chloroform:isoamyl alcohol (24:1) was added to the CTAB mixture and centrifuged at 3000rpm for 10 minutes. The aqueous phase was extracted and placed into a fresh falcon tube and the chloroform extraction was repeated (3000rpm; 10 minutes). The aqueous phase was moved to a fresh falcon tube again and 4.2 ml of 100% isopropanol was added and mixed

by gentle inversion. The tubes were then centrifuged at 3000rpm for 5 minutes to gather precipitated DNA. The supernatant was discarded and the pellet washed with 70% ethanol before allowing the pellet to air-dry. The pellet was then resuspended in 650µl resuspension buffer, moved to a sterile 1.5 ml microcentrifuge tube, and incubated at 37°C for 30 minutes. A final chloroform extraction was performed using equal amounts of chloroform:isoamyl alcohol to the resuspension buffer and spun briefly. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and 650µl of isopropanol was added. The mixture was then centrifuged at 14000rpm for 5 minutes and the supernatant discarded. The pellet was washed in 70% ethanol and spun for 1 minute at 14000 rpm before being allowed to air-dry. The pellet was then resuspended in 100µl of T.E. buffer.

2.6.3 RNA extraction

The SV total RNA isolation kit (Promega) was used for all RNA isolations in plant and seed tissue.

Materials

SV RNA lysis buffer (Promega)

- 4 M guanidine thiocyanate
- 10 mM Tris-HCl (pH 7.5)
- 0.97% β-mercaptoethanol

SV RNA wash solution (Promega)

- 60 mM potassium acetate
- 10 mM Tris-HCl (pH 7.5)
- 60% Ethanol

RNA dilution buffer (Promega)

DNase solution:

- Yellow core buffer (Promega) (40 µl per spin column)
- MnCl₂ (5 µl per spin column)
- DNase I (5 µl per spin column)

DNase stop solution

Absolute ethanol

70% ethanol

sdH₂O

Method

RNA was purified and extracted on a silica spin column. Tissue was ground using a pestle and mortar in liquid nitrogen. Ground tissue was placed in a sterile microcentrifuge tube containing 175 µl RNA lysis buffer and 350 µl dilution buffer and mixed by gentle inversion. The solution was then incubated for 3 minutes at 70°C before centrifuging at 14 000 rcf for 10 minutes. The lysate was transferred to a fresh sterile microcentrifuge tube and 200 µl 95% ethanol added. This mixture was placed inside a spin column and centrifuged at 14 000 rcf for 1 minute and the flow-through discarded. 600 µl of wash solution was then added to the membrane and centrifuged at 14 000 rcf for 1 minute. After discarding the flow-through 50 µl of DNase solution was added directly to the spin column membrane and left to incubate at room temperature for 15 minutes followed by adding 200 µl of DNase stop solution and centrifuging at 14 000 rcf for 1 minute. 600 µl of wash solution was added to the column and centrifuged at 14 000 rcf for 1 minute and the flow-through discarded. A final wash with 250 µl of wash solution at 14 000 rcf for 2 minutes ensures the removal of residual buffers. To elute the DNA the spin column was placed into a fresh, sterile microcentrifuge tube and 50 µl of sdH₂O was added to the membrane before centrifuging at 14 000 rcf for one minute. Quantity and quality of RNA was evaluated on an ND-1000 spectrophotometer and the RNA was then stored at -80°C.

2.6.4 cDNA synthesis

cDNA synthesis was performed using the SuperScript II Reverse Transcription kit (Invitrogen) along with RNA from the previous section.

Materials

- 200 U µl⁻¹ SuperScript II Reverse Transcriptase (RT)
- 5x First strand buffer (FSB)
- 0.1 M DTT
- dNTP mix (dATP, dCTP, dGTP and dTTP, 10 mM each.)
- 500 µg ml⁻¹ Oligo-deoxythymidine (Oligo (dT))

Method

To synthesise DNA typically 200-500ng of total RNA was added to a 0.2 ml centrifuge tube along with 1 µl Oligo (dT) and 1 µl dNTP mix, made up to 12 µl with sdH₂O. The tubes were incubated at 65°C for 5 minutes and then chilled on ice. Following cooling 4 µl of FSB, 2 µl of DTT and 1 µl of RT were added to the centrifuge tubes and incubated at 42°C for 50 minutes. The RT was then inactivated by heating at 70°C for 15 minutes. cDNA was stored at -20°C.

2.7 DNA amplification via PCR reaction

2.7.1 DNA amplification

PCR amplification of DNA fragments were conducted using GoTaq (Promega) utilising *Thermus aquaticus* Taq DNA polymerase as described below.

Materials

GoTaq® Green Master Mix, Promega (2x):

- 2x Green GoTaq® Reaction Buffer (Ph 8.5)
- 3 mM MgCl₂
- dNTP mix (0.4 mM each of: dATP, dCTP, dGTP and dTTP)
- 50 U ml⁻¹ Taq DNA polymerase
- 10µM Oligonucleotide primers
- T.E. buffer

Method

Oligonucleotide primers were designed to have between 20-25bp homology with the target sequence to be amplified and generated a fragment of DNA of specific size dependent on subsequent analysis. A 100µM stock of each primer was made using T.E. buffer, subsequently diluted in sdH₂O to generate a 50x solution (10µM). PCR reactions using the 2x GoTaq® Green Master Mix were used at a final concentration of 1x following the addition of 50-500ng DNA, sdH₂O and 0.5µM each of forward and reverse primers. The reactions took place in 0.2 ml PCR tubes with a 20 µl final volume. The reactions took place in a SensoQuest LabCycler thermocycler for standard PCR and a BioRad CFX Connect cycler for qPCR using the programmes described below. Annealing temperatures (highlighted in bold) were altered slightly (+/-3°C) depending on primer pair T_m and PCR product length.

2.7.2 PCR programmes

95°C 1 minute

95°C 20 seconds

53°C 20 seconds

72°C 60 seconds

} 20-30 cycles dependent on copy number

72°C 5 minutes

2.7.3 RT-PCR programmes

95°C 1 minute

95°C 20 seconds

53°C 20 seconds

72°C 60 seconds

95°C 10 seconds

} 50 cycles.

Melt curve: 65°C rising in 0.5°C every 5 seconds up to 95°C.

The plate in RT-PCR programmes is analysed for copy number after each cycle.

2.8 Enzymatic Digestion of DNA

2.8.1 Uracil DNA glycosylase treatment of DNA

Materials

DNA sample

10x UDG reaction buffer (New England BioLabs)

UDG (New England BioLabs)

sdH₂O

Method

DNA (~1ng) was kept on ice and made up to 8 µl with sdH₂O. 1 µl of UDG and the same amount of Buffer 1 were added to the mixture and incubated at 37°C for 3 hours. A control reaction that did not excise 8-oxoG underwent the same

treatments but did not contain UDG, instead having 1 µl of sdH₂O. The reaction was stopped by heating at 95°C for 10 minutes.

2.8.2 FPG treatment of DNA

Materials

DNA sample

Buffer 1 (New England BioLabs)

FPG (New England BioLabs)

sdH₂O

Method

DNA (~1ng) was placed on ice and adjusted to 8 µl with sdH₂O. 1 µl of FPG and the same amount of Buffer 1 were added to the mixture and incubated at 37°C for 3 hours. A control reaction underwent the same treatments but did not contain FPG, instead having 1 µl of sdH₂O. The reaction was stopped by heating at 60°C for 10 minutes.

2.9 Gel electrophoresis

2.9.1 Agarose gel electrophoresis

Materials

Tris, boric acid, EDTA (TBE) buffer:

- 89 mM Tris base
- 89 mM Boric acid
- 2 mM EDTA

Agarose

GelRed (Biotium)

Loading buffer:

- 50% (v/v) Glycerol
- 0.05% (w/v) Bromophenol blue
- 0.05% (w/v) Xylene cyanol

Hyperladder 1kb plus (Bioline)

Method

For smaller cast gels, agarose was added to 40 ml TBE buffer and for larger cast gels agarose was added to 100 ml TBE buffer inside an open topped beaker. The amount of agarose added was dependent on predicted product size. The standard percentage agarose in the gel was 1.0% which was lowered accordingly for smaller products.

The beaker was microwaved until the agarose had dissolved. The gel was either left to set in a gel cast with comb (BioRad) awaiting post-electrophoretic staining or was left to cool to 65°C and GelRed (5 µl/100 ml of TBE) was added before being poured and left to set. Once set, the gel was placed in an electrophoresis tank (BioRad) and overlaid with TBE buffer. If the samples did not already contain a loading dye 0.25 volumes of loading buffer were added. The samples were then loaded into the wells alongside Hyperladder I kb plus (5 µl) to verify size of products. Electrophoresis was conducted at a constant voltage to allow DNA migration through the gel.

2.9.2 Alkaline agarose gel electrophoresis

Materials

Agarose

10x Alkaline agarose gel electrophoresis buffer (electrophoresis buffer):

- 500 mM NaOH
- 10 mM EDTA

Absolute ethanol

70% ethanol

Staining solution:

- GelRed (30 µl/100 ml TBE)
- 1x TBE buffer

Neutralizing solution:

- 1 M Tris-Cl (pH 7.6)
- 1.5 M NaCl

Tris, boric acid, EDTA (TBE) buffer:

- 89 mM Tris base
- 89 mM Boric acid
- 2 mM EDTA

Method

For smaller cast gels agarose was added to 40 ml sdH₂O and for larger cast gels agarose was added to 100 ml sdH₂O inside an open topped flask and heated in a microwave until dissolved. After the mixture had cooled to 55°C 0.1 volumes of 10x electrophoresis buffer was added. Once the gel had set it was placed inside an electrophoresis tank (BioRad) and overlaid with 1x electrophoresis buffer. The DNA samples were mixed with 0.2 volumes of alkaline gel loading buffer and loaded into the wells before running at a constant 3.5 V cm⁻¹ of gel. To stain the gel it was first neutralized by gentle mixing in neutralizing solution for 45 minutes. The neutralizing solution was rinsed from the gel with TBE and the gel was submerged in staining solution for one hour before imaging and analysis.

2.9.3 Urea poly-acrylamide gel electrophoresis (Urea PAGE)

Urea PAGE gels were performed using reagents from the Sequagel Ureagel system protocol (National Diagnostics (ND)) according to the manufacturer's instructions.

Materials

UreaGel concentrate (ND):

- 237.5 g l⁻¹ Acrylamide
- 12.5 g l⁻¹ Methylene bisacrylamide
- 7.5 M Urea

Ureagel buffer (ND):

- 0.89 M Tris-borate
- 20 mM EDTA (pH 8.3)
- Urea

Loading buffer:

- 90% Formamide

- 0.5% EDTA
- 0.1% Xylene cyanol
- 0.1% Bromophenol blue

Ureagel diluent (ND)

Tetramethylethylenediamine (TEMED)

10% Ammonium persulfate (APS) freshly made

Method

Urea PAGE gels were created by adding 20 ml UreaGel concentrate, 70 ml UreaGel diluent and 10 ml UreaGel buffer in a glass beaker. In order to initiate gel formation 40 µl of TEMED and 800 µl of freshly prepared 10% APS were added. The mixture was poured into two gel casting stands (Mini-protean; BioRad) and combs placed on top. Once the gels had polymerised they were added into an electrophoresis chamber (Biorad) and submerged in 1x TBE and connected to a standard power pack. The gel was then pre-run for 30 minutes at constant voltage to warm the gel and remove excess urea. The wells were then rinsed briefly in TBE to remove standing urea that may affect DNA upon loading. Loading buffer was added to the samples and they were added to wells, any empty wells contained only loading buffer to maintain equal conditions across the acrylamide gel. The gel was run at a constant voltage until the dye had reached the bottom of the gel. Once complete the gels were rinsed in TBE and visualised and processed using software as mentioned in section 2.2.

2.9.4 DNA purification using agarose Gels

Gel extraction was performed using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

Materials

Buffer QG (Qiagen)

Buffer PE (Qiagen)

3 M Sodium acetate (pH 5.0)

Absolute isopropanol

sdH₂O

Method

The protocol uses spin columns with silica membranes to purify DNA. The desired fragment of DNA was extracted from the agarose gel using a scalpel and weighed in a 1.5 ml microcentrifuge tube. Binding and solubilisation buffer (Buffer QG) was added (300 µl per 100 mg of gel fragment) and incubated at 50°C until the gel was dissolved and the solution turned yellow. If the solution did not turn yellow, 10 µl of sodium acetate (pH 5.0) was added. One gel volume of absolute isopropanol was added, followed by mixing by gentle inversion, in order to precipitate the DNA. The precipitated DNA was bound to a QIAquick spin column silica membrane by centrifugation at 12 000 rcf for one minute. The column was then washed by adding buffer PE and centrifuging 12 000 rcf for one minute. This step was repeated before adding 30 µl of sdH₂O and centrifuging for 2 minutes at 12 000 rcf in order to elute the DNA from the column.

3. Characterisation of EcoSeed seed lots

3.1 Introduction

3.1.1 Seed quality

Seeds can be classified into three categories; orthodox, recalcitrant and intermediate species. Following seed maturation, orthodox seeds undergo maturation drying, reducing both water content and metabolic activity and allowing them to survive for extended periods before germinating (Ellis et al., 1991). *Arabidopsis thaliana* and *Brassica oleracea*, the species used in this study, are both orthodox (desiccation tolerant) seeds.

Orthodox seeds, representing most agricultural species, are stored in a variety of circumstances dependent on the global range of agricultural practices. Elevated heat and humidity increases the speed of deterioration of macromolecules (Waterworth et al., 2015). Deterioration is particularly problematic in the developing world with limited access to controlled storage facilities. This deterioration includes an increase in the levels of genome damage apparent in aged seeds (Cheah and Osborne, 1978). The natural deterioration of seeds in storage can be simulated during accelerated ageing (AA) protocols: subjecting the seeds to elevated heat and humidity for short periods of time mimics natural seed ageing (Powell and Matthews., 2012; Rajjou et al., 2008). The related method of controlled deterioration (CDT) is optimised for larger seeds, whereby seeds are fully equilibrated to defined hydration levels before they are subject to heat-induced ageing (Mavi and Demir, 2007). Both methods allows for in depth analysis on seed damage and repair during ageing to help uncover the molecular basis that underpin differences in seed vigour and viability (Powell and Matthews, 2012). These two important aspects of germination determine the quality of a seed lot.

Successful germination is crucial for plant survival and a key determinant of crop yields. Reduction in seed quality can cause considerable problems in agriculture and conservation efforts in wild species, leading to considerable economic loss. Two factors that strongly affect the quality of a seed are maternal environment (Zas et al., 2013) and storage following desiccation (Powell and Matthews, 2012; Waterworth et al., 2015).

3.1.2 The EcoSeed project overview

To date, despite the importance of seed quality to ecology and agriculture, we know surprisingly little about the molecular processes that determine the speed of germination and loss of seed viability. The work in this thesis forms a component of the EcoSeed EU Horizon 2020 funded project which, combining the expertise of agricultural industry experts, seed conservation scientists and several EU-wide universities, aimed to increase our understanding of the basis of seed quality. A key aim of the project was to determine the factors that underpin seed quality, in terms of molecular changes within seeds and in relation to their maternal environment. Through delineating the molecular changes that occur in the seed following different stresses in the dry seed and during imbibition, the project aimed to identify the important changes that affect seed quality through development, storage and throughout imbibition. In addition, an integral part of the EcoSeed project was the inclusion of crop species in addition to the model plant *Arabidopsis thaliana*. Application of the project findings, through identification of genetic determinants of seed quality, would provide candidate biomarkers for seed quality in Brassica and barley. The ideal marker for seed quality would be transferable, cost efficient, high throughput relatively simple and fast to undertake. Applying all of these traits generates seed quality biomarkers that can be widely accessible and utilisable in agriculture, conservation and assist in plant breeding programmes.

3.1.3 The EcoSeed project: Leeds

The objective of the work presented in here was to analyse the relationship between seed quality and the types and levels of DNA damage in seeds, along with an analysis of the DNA damage response and its role during early imbibition and germination. Long periods of time cause seeds to lose viability and vigour associated with ageing and oxidative stress, with higher temperatures and humidity causing accelerated decline (Goel et al., 2003; Powell and Matthews, 2012). The desiccated state in dry, orthodox seeds allows survival through harsh environmental conditions, in some species successfully propagating after centuries (Shen-Miller et al., 1995). The desiccation/rehydration cycles that orthodox seeds often go through before

initiating germinative processes are associated with increased levels of damage to macromolecules and cellular structures (Rajjou et al., 2012). In conjunction with low levels of metabolic activity coupled with low levels of respiration allows for damage to accumulate without repair or scavenging systems to generate a repair response leading to a reduction in seed vigour and viability. During storage DNA breaks and other forms of damage accumulate, contributing to the drop in overall seed performance (Dourado and Roberts, 1984; Waterworth et al., 2016). This damage to DNA induced by storage has been shown to occur across a range of species (Cheah and Osborne, 1978; Dourado and Roberts, 1984; Dandoy et al., 1987; Liu et al., 2004; El-Maarouf-Bouteau et al., 2011). If DNA damage accumulates to cytotoxic levels, seeds will eventually lose the capacity to germinate and the seed will die (Kranner et al., 2010).

3.1.4 Seed vigour: a measure of seed quality

Uptake of water by the seed initiates the start of germination and completion is determined by the protrusion of the radicle from the seed coat (Bewley and Black, 1994). The speed at which a seed completes germination is defined as seed vigour and is an important determinant of how productive the plant will be (Rajjou et al., 2012). Low vigour seeds produce weaker seedlings that are more prone to influence from environmental conditions and typically show a reduction in yield (Ellis et al., 1990). Seed vigour is influenced by several factors including environmental conditions during development, storage conditions and exogenous factors such as nutrient availability and pathogen presence (Rajjou et al., 2012). The most adverse conditions result in reduced seed viability with accumulated damage causing cell death.

Current methods of determining seed quality differences rely on germination testing samples of seeds to determine vigour and viability. Drops in viability of seed lots are usually associated with a large accumulation of damage and are more uncommon in agricultural practices. Vigour differences are more common and result in reduction of crop yield. Seed vigour can be portrayed as the mean germination time (MTG) of a seed lot and calculated using equation 3.1. Natural ageing generates differences in seed quality which can be simulated by raising the moisture content of seeds, known as controlled

deterioration. Similarly vigour differences can be generated using specified higher temperatures and relative humidity over a shorter period of time, known as accelerated ageing (Powell and Matthews, 1984). The reliance on germination testing opens the door for novel methods of predicting seed quality, with seed vigour a candidate for predicting crop yield allowing for the generation of quantitative, sensitive biomarkers to evaluate seed quality.

3.1.5 The significance of seed quality

Economic losses are suffered every year by farmers due to poor seed quality. This is a particular problem in developing areas of the world with restricted access to controlled storage facilities and hot, humid climates. Global warming is set to further exacerbate problems with agriculture and wild-species conservation centres bearing the brunt of reduction in seed quality and crop yield. This increases the importance of the generation of universal markers for seed quality that can be utilised across a number of species. Using accelerated ageing to produce seeds with differences in vigour, we look to develop our understanding of how DNA damage affects seed vigour and potentially look for effective biomarkers to estimate vigour differences.

$$\bar{t} = \frac{\sum_{i=1}^k n_i t_i}{\sum_{i=1}^k n_i}$$

Mean germination time equation

t_i is the start of the experiment, post stratification, to the i^{th} observed time. n_i is the number of seeds to germinate in the i^{th} time interval and k is the point in which the last seed has germinated. The average of each independent test gives the final MGT value.

Seed quality is of great importance to agriculture, conservation and food security. The added challenge of increasing global temperatures require new knowledge on how rapidly changing climates will affect seeds during development and storage, in order to better predict quality of seed batches and minimise losses. Correlating the levels of DNA damage and the cellular responses DNA damage with seed quality would strengthen the link between these processes, in addition to identifying candidate biomarkers. Thus, the

germination characteristics of seeds grown in different environments and storage conditions, in terms of vigour, viability and storability can be correlated with a molecular analysis of genome integrity. Seed lots were produced by the EcoSeed network or at Leeds, under standardised conditions used by all project partners. This section will assess the quality of seed lots used in during the project by germination testing. Chapters 3-7 report the analyses of damage accumulation and repair in the seed lots and relate these factors to the germination profiles reported here.

3.1.6 Species studied in EcoSeed seed lots

Two plant species were used in the project-the model plant *Arabidopsis thaliana* (hereon referred to as *Arabidopsis*) and the closely related UK crop *Brassica oleracea* spp. *oleracea* (hereon referred to as *Brassica*). The former provides us with a large genetic resource, including a sequenced genome and a wide variety of knock-out mutants available. Brassica allows for the translation of the knowledge into crop species and comparison of the relationship between genome maintenance processes and seed quality across different species. The *Brassica* genus includes a number of crop species such as broccoli, cabbage, cauliflower, kale and sprouts. *Brassica oleracea* has a sequenced pangenome; unlike sequencing individual organisms a pangenome allows the incorporation of structural variations, such as presence/absence variance and copy number differences, which are characteristic of *Brassica* species (Golicz et al., 2016).

3.1.7 Aims

The aim was to analyse the germination characteristics of the EcoSeed seed lots to determine the effects on vigour and viability resulting from the seeds produced under a range of maternal environmental temperatures and subject to different ageing regimes. This characterisation then allows for the comparison of seed quality with downstream analysis of DNA damage, repair and oxidation in further chapters. Two different *Arabidopsis* seed lots were used in this studies, one generated at the University of Leeds that were subjected to accelerated ageing (section 3.2.1) and *Arabidopsis* and *Brassica*

seeds produced at Warwick University for use in the EcoSeed project (section 3.2.2).

3.2 Results

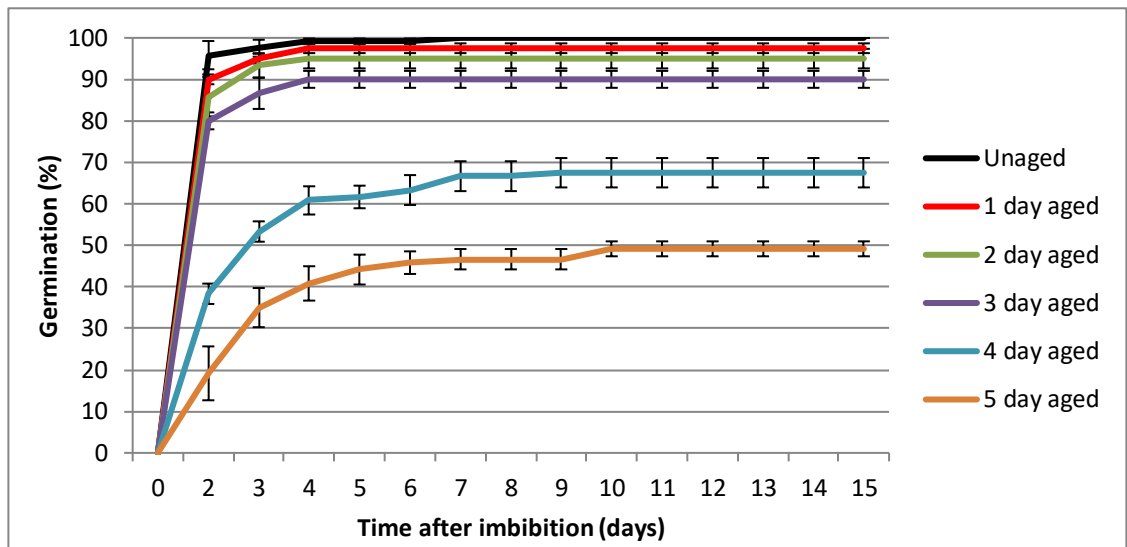
3.2.1 Accelerated ageing of *Arabidopsis thaliana* seeds

Two different seed lots of *Arabidopsis* were used in this work. This section reports data obtained using seed lots generated and subjected to accelerated ageing at the University of Leeds. To obtain seed lots, plants were propagated from seeds that were surface sterilised and plated onto MS medium before being placed into a control temperature room (22°C; 16 h:8 h day/night cycle). After two week's growth, seedlings were transferred to soil and placed in greenhouses, as described in (section 2.4). Mature plants that had set seed were allowed to dry once all siliques had turned brown. Harvested seed were stored for two months at room temperature to allow after-ripening. These are referred to a 'high quality' seed to distinguish them from seeds subject to accelerated ageing.

The high quality seeds produced were of 100% final viability, and uniform germination, on average showing radicle protrusion within 50 hours of the onset of imbibition (Figure 3.1). To generate seeds of lower quality, seeds were deteriorated by accelerated ageing. The ageing regime involves seeds incubation above a saturated solution of potassium chloride at elevated temperature in an air-tight vessel (35°C; 80% relative humidity) (Hay et al., 2003; Powell and Matthews, 2012). Viability and vigour are reduced dependent on the time period seeds are deteriorated. Seeds were deteriorated in the high temperature/humidity environment for between 1 and 5 days, with samples being removed every 24 hours to generate seed lots of progressively deteriorating quality (0 d, 1 d, 2 d, 3 d, 4 d and 5 d aged). Monitoring germination over a time course allows calculation of both final germination percentage and the average mean it takes for the seeds to germinate. Germination tests are performed as described in section 2.5.2. Analysis of germination, including calculation of mean germination time (MGT) is described in section 2.5.2.

Seed viability was 97.5% and 95% respectively for seeds aged for 1 and 2 days. Seeds began to lose significant viability following ageing for three days when final germination dropped to 90% ($P < 0.05$). The mean germination time (MGT) showed no significant change up to 3 days ageing, averaging between 50 and 52 hours. Following 4 days of ageing, there was a drop in both viability and vigour with final germination percentage dropping to 67.5% and the mean germination time rising to 66 hours. Further decline was seen in seeds aged for 5 days, with only 49% of seeds germinating. Of those seeds that did germinate, radicle emergence was greatly delayed; on average it took 88 hours for the protrusion of the radicle from the seed coat (Figure 3.1).

[A]



[B]

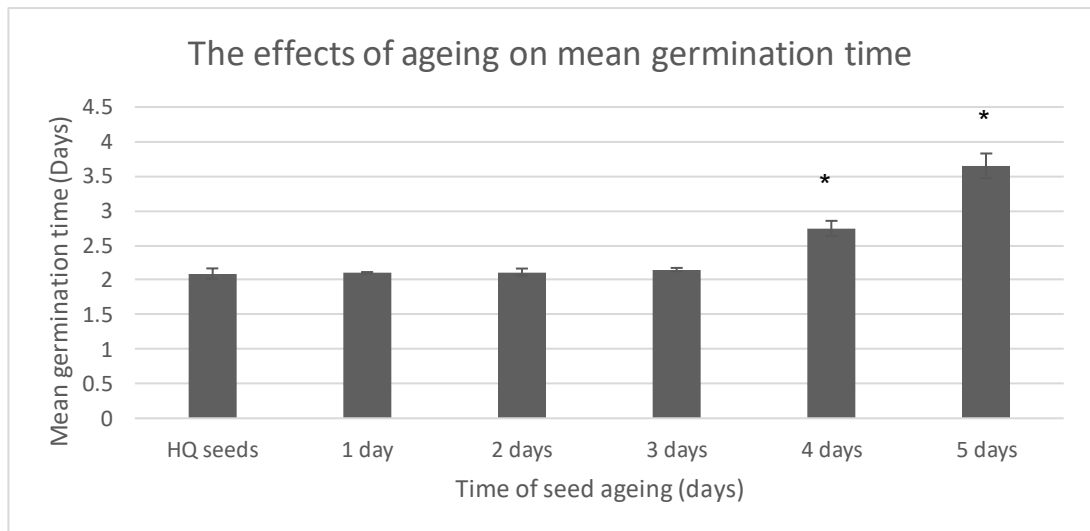


Figure 3-1 Germination performance of *Arabidopsis* seed

Seeds were grown as described in section 2.4 at the University of Leeds, subjected to ageing (section 2.5.3) (35°C; 80% RH) and germinated on germination paper. Germination was scored every 24 hours (section 2.5.2).

[A] Daily germination percentages of *Arabidopsis thaliana* seeds across different accelerated ageing regimes.

[B] Mean germination time (MGT) of *Arabidopsis thaliana* seeds. The average time it takes for a seed to germinate is an indication of vigour, with highest quality *Arabidopsis* seeds germinating within 2 days.

3.2.2 The effects of maternal environment on *Arabidopsis thaliana* seed germination performance

Seed lots were produced at the University of Warwick for the EcoSeed project for distribution to partners. EcoSeed seed lots underwent several different stresses, including either elevated, reduced or control temperature during seed maturation on the mother plant. In addition, accelerated ageing was performed on seed lots from each treatment to analyse the impact of the maternal environment on seed performance and stress responses. For *Arabidopsis* this consisted of three treatments: a low temperature stress (LT; 14°C/16°C), control temperature (CT; 18°C/22°C) and high temperature (HT; 25°C/28°C). In *Brassica* a control temperature (CT; 18°C/22°C) and high temperature (HT; 25°C/31°C) were used (table 3.1). The variable temperature regimes replicated, as closely as possible, predicted climatic changes to see how seed production and quality will be affected in the near future. During formation on the maternal plant, temperature drastically affected both seed quality and seed yield per plant. *Arabidopsis* and *Brassica* produced fewer seed in smaller siliques at higher temperatures. Thus seed production was very vulnerable to temperature stress in the maternal environment, and is known to cause losses in agriculture (Semenov et al., 2014).

This study aimed to investigate agriculturally relevant changes to seed quality associated with potential climate fluctuations and mild ageing treatments. This meant that seed lots with very viability were not included in this investigation, and that the combination of treatments generating small viability differences or changes in vigour were of most interest for the investigation of associated molecular changes in seed. Deterioration in storage was simulated using conditions of elevated temperature and relative humidity which were optimised for each species studied. In initial studies, the effects of hypoxia (atmospheres containing <3% O₂) were also investigated but there were no differences in the germination characteristics between seeds exposed to low oxygen atmospheres and controls. Controlled deterioration (CDT) for up to 11 days was used to generate a range of differences in seed quality in *Arabidopsis* seeds. For CDT, seeds were equilibrated at 20°C and 75% RH for 1 day and then transferred to 40°C and 75% RH. Interestingly,

seeds obtained from plants grown at a lower temperature (LT), which induces dormancy, lost this thermodormancy following CDT for 5 days. Utilising the different maternal environments and CDT stresses, significant differences in seed quality were obtained. These results allow both an investigation of the effects of the maternal environment on germination performance and also provide a resource allowing the relationship between DNA damage/repair and seed quality to be studied (Figure 3.2 and 3.3). *Brassica* seeds showed greater sensitivity to CDT than *Arabidopsis*, losing viability within 9 weeks of CDT, and reduction in vigour and viability apparent following 6 days at 45°C, 83% RH (Figure 3.3).

Germination analysis of the EcoSeed seed lots was performed at (15°C; 24 h day) in growth chambers, as this was required to relieve thermal dormancy induced in seeds obtained from plants grown under LT conditions. An additional advantage of analysing seed performance at lower temperatures was that slowed germination accentuated differences in seed lot vigour.

LT	Seeds grown in a low temperature environment during development. 14°C-16°C; 16 hour days.
CT	Seeds grown in a control temperature environment during development. 18°C-22°C; 16 hour day
HT	Seeds grown in a high temperature environment during development. 25°C-28°C; 16 hour day

LTA CTA HTA	An "A" suffix denotes controlled deterioration of the above seeds at 40°C with 75% relative humidity for 4 days (e.g. LTA, CTA, HTA)
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Table 3.1 Notations for the environmental conditions while *Arabidopsis* seeds were developing on the maternal plant and post-harvest ageing conditions

In unaged seeds, final germination of LT, CT and HT seeds were 91.7%, 97.5% and 99.16% viability respectively. There was no change in viability observed between CT seeds and either LT ($P=0.24$) or HT ($P=0.52$) seeds; however HT seeds had the highest level of viability (rather than control temperature seeds) and was significantly greater than that of LT seeds ($P<0.01$). Seed lot viability reduced significantly following ageing of LT and CT seeds, with seed viability dropping to 57.5% and 75.8% in LTA ($P<0.01$, aged vs unaged) and CTA ($P<0.05$) seeds. The controlled deterioration of HT seeds produced no significant change (84.2% from 99.1% final germination; $P>0.05$). Seeds produced under lower temperature conditions, displayed greater sensitivity to CDT compared to the control or high temperature (Figure 3.2).

The mean germination time (MGT) of the seed lots, a measure of vigour, reflected the loss in viability. CDT increased the mean germination time following ageing in all three conditions. LT seeds took 110.8 hours on average to germinate. This was significantly longer than CT (86.4 hours) or HT (67.0 hours) ($P<0.05$). HT seeds germinated the fastest, displaying increased vigour in comparison to the CT seeds. Similarly LTA seeds took significantly longer (163.9 hours) than CTA (127.4 hours) and HTA (127.0 hours) seed. However no significant differences were between CTA and HTA seeds were observed.

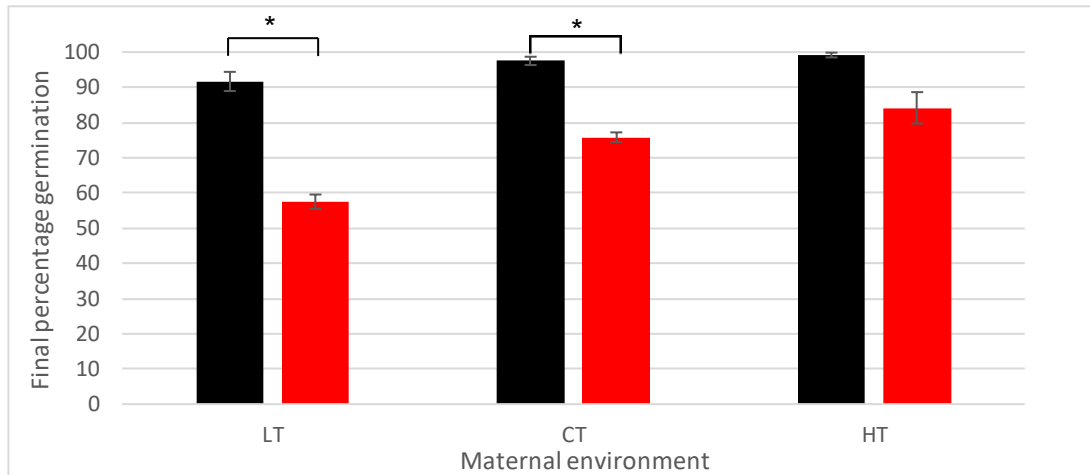


Figure 3.2 Germination percentages of aged and unaged EcoSeed *Arabidopsis* seed lots produced under different maternal environments

Germination percentages of *Arabidopsis thaliana* seeds grown at different maternal temperatures and either kept as high quality controls (■) or subjected to CDT (controlled deterioration) ageing regimes (■). Ageing lowered the viability in seeds grown in low or control temperature regimes (* = $P < 0.05$) but not in the seeds grown at higher temperature. Maternal environment conditions: **LT**; 14°C-16°C, **CT**; 18°C-22°C, **HT**; 25°C-28°C. CDT of seeds undertaken at 40°C with 75% relative humidity for 4 days.

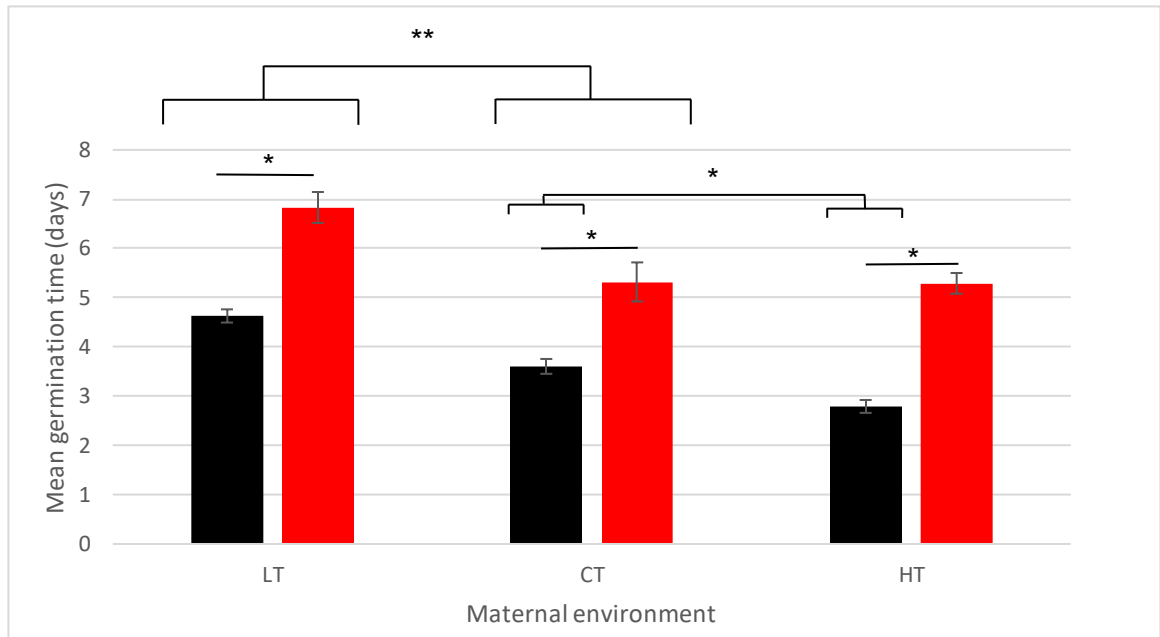


Figure 3.3 Mean germination time (MGT) of aged and unaged EcoSeed *Arabidopsis* seed lots produced under different maternal environments

The mean germination time (MGT) for *Arabidopsis thaliana* seeds grown at different maternal temperatures and either kept as high quality controls (■) or subjected to CDT ageing regimes (■). CDT causes an increase in the amount of time taken for the seeds to germinate i.e. a reduction in vigour ($P < 0.05$ for all conditions). Seeds grown in a low temperature environment, with or without ageing, take longer on average to germinate than those grown at the control temperature ($P < 0.01$). Seeds grown at a higher temperature germinate faster than those kept in the control temperature ($P < 0.05$) but only in high quality seeds. P-values given from Student's T-Test with 3 replicates of 40 seeds for each condition, * = $P < 0.05$, ** = $P < 0.01$. Maternal environment conditions: **LT**; 14°C-16°C, **CT**; 18°C-22°C, **HT**; 25°C-28°C. CDT of seeds undertaken at 40°C with 75% relative humidity for 4 days.

3.2.3 The effects of maternal environment and accelerated ageing on *Brassica oleracea* germination performance

Seed lots were produced at the University of Warwick for the EcoSeed project for distribution to partners. Similar to *Arabidopsis* seeds, *Brassica* seed lots underwent different temperatures during seed maturation on the mother plant. These conditions did not include a lower temperature, but a higher temperature was used (HT; 25°C/31°C) along with a control temperature (CT; 18°C/22°C). In addition to the maternal temperatures accelerated ageing was also used with both seed lots being subjected to high heat and humidity (45°C/85% RH) to simulate long term storage. The elevated maternal temperature regime was used to best replicate the future effects global warming may have on the quality of seed lots.

Brassica seeds were subjected to control and higher temperatures during seed development and subjected to ageing. There were no significant differences in viability between seeds grown in different maternal environments in unaged or aged seeds. CT seeds showed a 96.7% final germination percentage which lowered to 90.0% following ageing, although not significantly. The MGT for these seeds was 67.9 hours and 102 hours respectively. Despite the longer time it took to germinate for the aged seeds the differences were not significant ($P>0.05$).

Higher maternal temperature showed a reduction in viability, reduced to 56.7% following ageing when compared to unaged HT seeds which showed 98.3% viability. These HTA seeds germinated after 114.4 hours compared to HT seeds which generally germinated after around 72.0 hours, although this difference was again not significant ($P>0.05$).

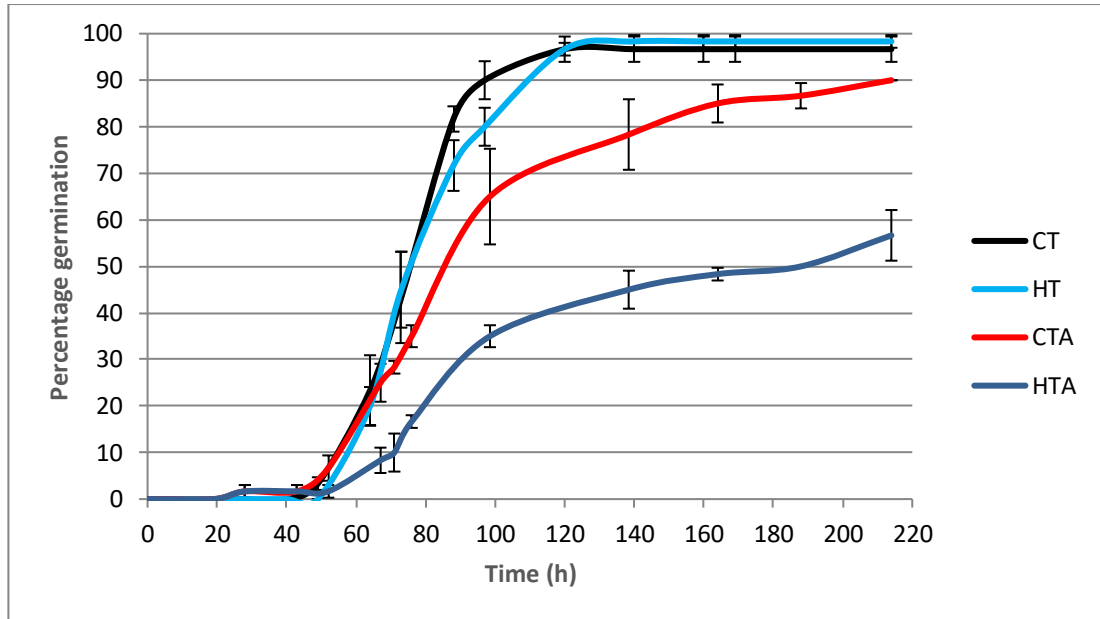


Figure 3.4 Germination of aged and unaged EcoSeed *Brassica oleracea* seed lots produced under different maternal environments

Germination percentages of *Brassica* seeds grown at different maternal temperatures and either kept as high quality controls or subjected to CDT ageing regimes. Ageing lowered the viability in seeds grown in high temperature regimes, with 98.3% of HT seeds germinating compared to 56.7% upon ageing (HTA)($P < 0.05$). There was no significant difference in final germination CT (96.7% germination) and CTA seeds (90.0%)($P = 0.18$). P-values given from Student's T-Test with 3 replicates of 35 seeds for each condition, * = $P < 0.05$, ** = $P < 0.01$. Maternal environment conditions: **CT**; 18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 24°C with 75% relative humidity for 10 days and is denoted by the suffix "A" (i.e. CTA and HTA respectively).

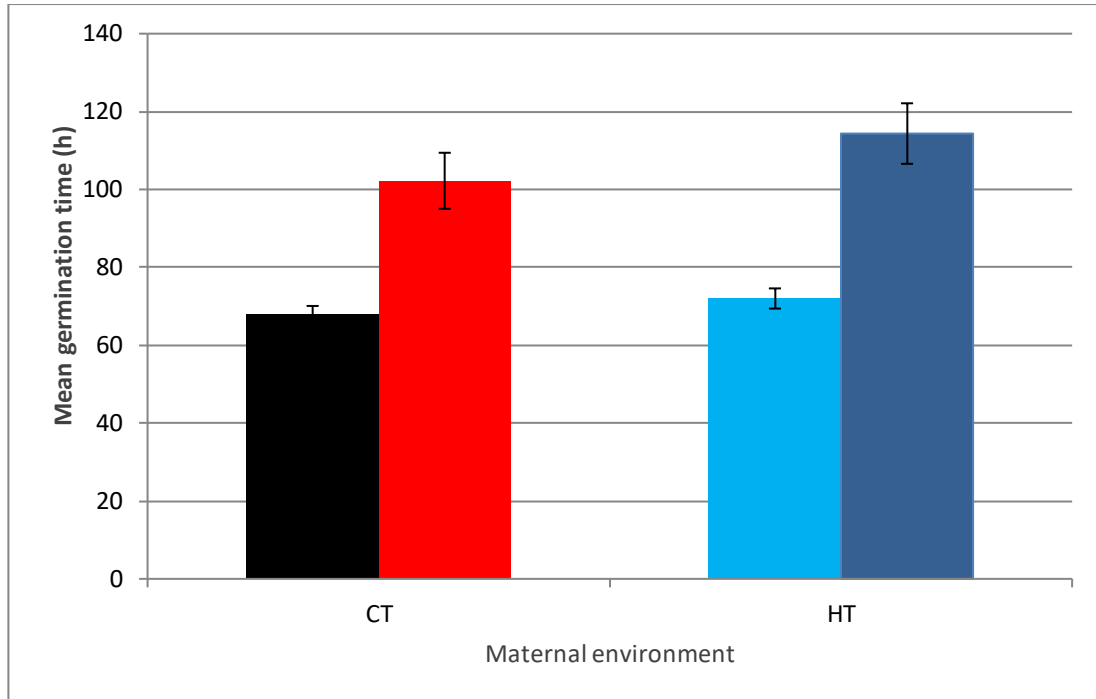


Figure 3.5 Mean germination time (MGT) of aged and unaged EcoSeed Brassica seed lots produced under different maternal environments

The mean germination time (MGT) for *Brassica oleracea* seeds grown at different maternal temperatures and either kept as high quality controls (■/▣) or subjected to CDT ageing regimes (■/▣). Maternal environment had no effect on MGT with CT seeds (67.9 hours) and HT seeds (72 hours) germinating at similar times. CTA (102 hours) and HTA seeds (114 hours) also did not differ from each other. There was no significant difference between CT and CTA seeds ($P=0.068$) nor any difference between HT and HTA seeds ($P>0.05$). P-values given from Student's T-Test with 3 replicates of 35 seeds for each condition, * = $P<0.05$, ** = $P<0.01$. Maternal environment conditions: **CT**; 18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 24°C with 75% relative humidity for 10 days and is denoted by the suffix "A" (i.e. CTA and HTA respectively)..

3.3 Discussion

3.3.1 Accelerated ageing can be used to mimic long term storage and lower the quality of seeds

A reduction in germination vigour precedes loss of viability and leads to losses in crop production through weaker seedlings and smaller yields (Ellis et al., 1990). Low vigour seeds and poor germination performance are associated with weak seedlings (low seedling vigour) reducing seedling establishment, plant productivity and yield (Finch-Savage and Bassel, 2015b). Here, the effects of the maternal environment on germination performance and seed longevity were analysed. In addition, these well-characterised seed lots provided the resource material used to determine the link between germination performance and genome damage. In commercial agriculture seed lots are required to have a minimum of ~90-100% viability when tested under optimal germination conditions. Therefore, an analyses of seed lots demonstrating significant loss in viability is less relevant to agricultural seed production, whereas reduction in vigour is a more useful and sensitive indicator of seed lot quality. Controlled deterioration (CDT) resulted in a small reduction in viability in *Arabidopsis* seed lots (Figures 3.1A and 3.2). Vigour differences are also seen following accelerated ageing as an accompaniment to the loss in viability (Figures 3.1B and 3.3).

3.3.2 The effects of the maternal environment

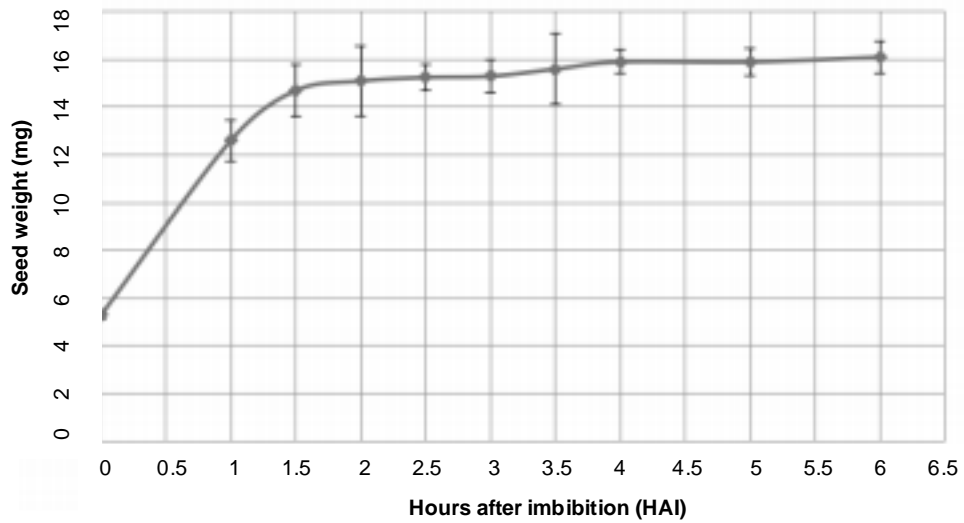
In this study seeds produced under low temperature maternal environments displayed reduced vigour in the absence of CDT (Figure 3.3). This may reflect residual levels of thermodormancy in these seed lots, as seeds grown in lower temperatures have been shown to remain dormant, even in ideal growing conditions (Bentsink and Koornneef, 2008). Seeds obtained from plants grown under higher temperature regimes in *Arabidopsis* displayed faster germination than those at the control temperature, with a significant increase in vigour, but no differences in viability observed between seed lots. This increase in vigour relative to controls was no longer evident after ageing. Thus, although seeds produced at a higher maternal temperature (HT) displayed higher vigour

immediately following after-ripening, no vigour differences were apparent between aged control (CTA) and HTA lines.

3.3.3 Germination data provided from the EcoSeed project partners

The first time-point selected for analyses in seed imbibition was an early imbibition time-point, aimed to assess the relationship between damage caused by an influx of ROS during imbibition and the repair processes that are initiated upon restarting of respiration. Early seed imbibition has already been shown to activate several responses associated with repair, including the DNA damage response (detailed in section 1.6) (Waterworth et al., 2010). The early time point was selected in order to ensure that the analysis of the early phase of germination would include repair activities. Six hours imbibition in *Arabidopsis* and 10 hours in *Brassica* were selected as these times represented when water uptake had reached a steady level (end of phase I of germination) (EcoSeed partner Finch-Savage lab, University of Warwick; Figure 3.4). Although water uptake seemed stable following 2-2.5 hours' imbibition in *Arabidopsis* this was deemed insufficient time for metabolic activities to be initiated and quantified. The late imbibition time-point was chosen to determine how much repair had occurred prior to germination initiation. The time-point in this case was taken as 80% of the time taken to reach germination. In the case of *Arabidopsis* this was 72 hours and 40 hours in *Brassica*.

[A]



[B]

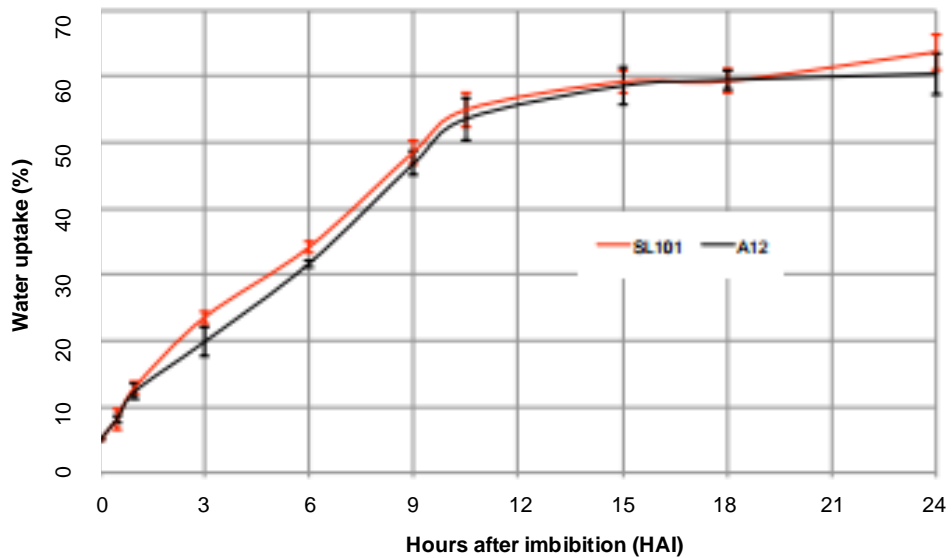


Figure 3.4 Water uptake in *Arabidopsis* and *Brassica*

Seeds were dry-blotted and weighed at different points throughout imbibition to determine when water uptake had stopped, signalling the end of phase I in germination. The weights were taken in *Arabidopsis thaliana* [A] and *Brassica oleracea* [B]. Two ecotypes of *Brassica* were utilised in initial experiments; SL101 and A12, with only SL101 being taken forward for further analysis due to residual dormancy present in A12 lines. Work undertaken by the University of Warwick.

The seed lots analysed here, with progressively reduced vigour and viability, form the basis of studies to investigate the molecular events important to seed quality, providing insight into the factors affecting seed performance. The following chapters will focus on the relationship between genome maintenance and germination performance integrity by analyses of lesions, levels of damage and DNA responses in seeds of different quality, from storage through to completion of germination.

4. Analysis of DNA integrity in seed ageing and germination

4.1 Introduction

DNA damage poses a serious threat to genomic stability, as it can lead to arrested growth, mutation and cell death. The maintenance of genomic integrity is essential for high seed vigour, yet DNA damage accumulates in the dry, quiescent state in the orthodox seed (Waterworth et al., 2016). This damage is apparent as an accumulation of single strand breaks (SSBs) associated with loss of seed viability, although the importance of different lesions on determining seed quality are unknown (Cheah and Osborne, 1978). DNA breaks can be caused directly through oxidation of the phosphodiester bond, connecting deoxyribose sugars in the DNA backbone. Breaks are also formed as an intermediate in excision repair pathways of damaged bases and nucleotides (Bray and West, 2005). Double strand DNA breaks (DSBs), representing broken chromosomes, are one of the most cytotoxic forms of DNA damage. DSBs can be formed through oxidation of both strands of the duplex which can occur during localised production of ROS caused by tracks of high energy radiation, or through the Fenton reaction mediated by heavy metal ions. It has been suggested that single strand breaks in close proximity can generate double strand breaks, and single stranded produced through the 'opening' in the DNA during repair is particularly sensitive to oxidation (Britt, 1996). Furthermore cells in the S-phase of the cell cycle can generate DSBs from single strand nicks as polymerases pass over the gap generated in the phosphodiester backbone, or through replication blocking lesions that lead to replication fork collapse (Lieber, 2010). Plants need to repair DNA damage upon seed imbibition, before cell cycle initiation and completion of germination, in order to prevent mutations (Waterworth et al., 2016). Preservation of meristem cells by delaying the cell cycle in response to genotoxins is important to plant growth under stress conditions (Heyman et al., 2013). DNA damage responses activated by the accumulation of strand breaks delay germination until sufficient repair has taken place (Preuss and Britt, 2003). To date, most focus has been on DSBs and less is known about the effects of SSBs in seeds.

4.1.1 Aims

The aim of this work was to develop a method to allow quantification of single strand breaks in seeds. This would then allow correlation between SSB accumulation and seed quality to be investigated. The approach taken was to quantify single DNA strand breaks using denaturing alkaline agarose gel electrophoresis. Under these conditions the duplex is denatured and single stranded DNA fragments migrate into the gel and can be quantified. In conjunction with current image analysis software, this provides a widely accessible, cheap and effective method of quantifying SSBs and predicting seed quality. The fraction of DNA that migrates into the gel can be quantified using GelRed in conjunction with image analysis software. This would then identify any correlation between levels of single DNA strand breaks and seed quality.

4.2 Results

4.2.1 Optimisation of genomic DNA extraction from *Arabidopsis* seeds

Because of the abundance of carbohydrates, seed coat mucilage, storage proteins, lipids and secondary metabolites in seeds, standard DNA extraction protocols are often ineffective. Impurities that co-purify with DNA make both spectrophotometric quantification and gel electrophoresis problematic. In order to circumvent the elution of contaminants (which display an absorbance peak at 230 nm), modifications to the standard plant DNA isolation procedures were required. Optimisation of extraction protocols involved adaptation of a CTAB-based extraction method in section 2.6.2. A higher salt concentration was used in the modified CTAB based extraction buffer, as initially NaCl was not present. Given the solubility of DNA in high salt solutions we raised this to 1.4 M. The incubation time during the initial extraction step was also reduced from 30 minutes to 2 minutes. Given the ability for DNA to dissolve rapidly in NaCl solutions this gave the highest chance to avoid contaminants from co-dissolving.

The concentration of NaCl used in the CTAB extraction buffer was varied originally to determine best conditions for DNA solubility. Concentrations varied from 0.4 M NaCl up to a maximum of 3.5 M NaCl and incubation time

was reduced from 30 minutes to 2 minutes. DNA remains soluble at the higher salt concentration but reduces levels of contaminants, whilst the reduced incubation temperature selectively reduces the time for hydration of seed coat mucilage. An optimised concentration of 1.4 M NaCl was used in subsequent extractions. The amount of starting material was found to be critical to the extraction of high quality DNA from *Arabidopsis* seeds. High amounts of powdered seed tissue resulted in high levels of co-purifying contaminants that co-purify with DNA, interfering with downstream analysis (Nonogaki, 2001). Because of the large differences in hydrated mucilage content of *Arabidopsis* seeds observed depending on level of imbibition (with low levels in dry seed and increased levels in seeds as they reach germination), different starting quantities need to be used for differentially imbibed seeds (table 4.1).

Table 4.1 Optimised starting quantities of freeze-ground *Arabidopsis* seed material at different time-points of seed imbibition

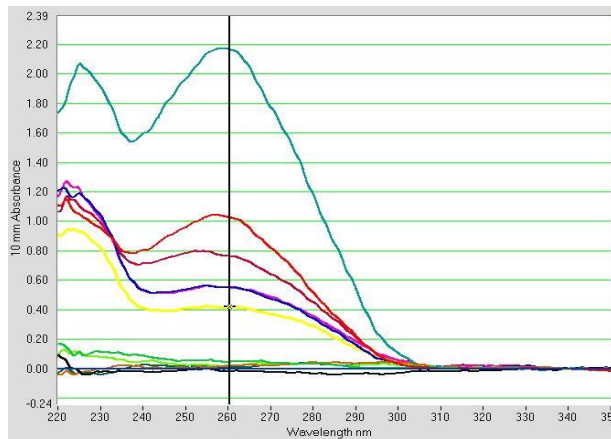
Imbibition time of seed	Quantity of starting seed material (mg)
Dry seed	20
Early imbibition (1-3 hours)	35
Mid stage imbibition (4-9 hours)	50
Late imbibition (10+hours)	75

Seeds such as *Brassica oleracea* have high levels of storage compounds; however most contaminants are lipid based and may be removed through standard chloroform extractions. Cereal seeds such as *Hordeum vulgare* required embryo dissection from the seed prior to DNA extraction to remove the starch enriched dead endosperm tissue which would otherwise interfere with DNA integrity analysis. In this case the standard CTAB extraction process produced high quality DNA with few contaminants (section 2.6.2).

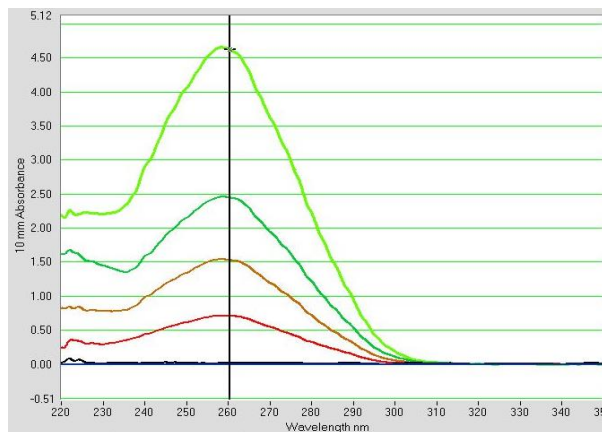
4.2.2 Single strand break analysis in Seeds

Single strand breaks pose a threat to genomic stability by blocking transcription and generating the highly cytotoxic double strand break when present at high levels or conversion of SSBs into DSBs during DNA replication (section 1.1.4) (Kuzminov, 2001). To enhance our understanding of how levels of single strand breaks correlate with the ability of a seed to germinate two approaches were developed to quantify the number of single strand breaks in genomic DNA isolated from *Arabidopsis* seeds: alkaline agarose and urea acrylamide gels. Both methods use a denaturing agent to disrupt the hydrogen bonds in duplex DNA. Single stranded DNA is then separated by either acrylamide or agarose gel electrophoresis. Lower molecular weight DNA migrating away from the intact genomic DNA and represented fragmented regions, can be quantitated by staining with GelRed and quantified using image analysis software.

[A]



[B]



[C]

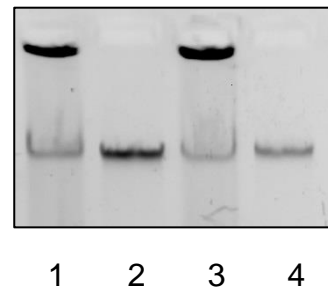


Figure 4.1 Optimisation of genomic DNA isolation from seeds: Quality difference of DNA extracted using different CTAB methods

DNA extracted from *Arabidopsis* seeds contain large amounts of contaminants at the 230nm spectrum when DNA is quantified using a spectrophotometer showing nucleic acids with a peak absorbance at 260nm.

[A] DNA extraction using the standard CTAB method for plant material. The standard method for extracting DNA from plant material (Doyle, 1987) contains contaminants which absorbed at 230nm in *Arabidopsis* seeds.

[B] DNA extraction with modified NaCl content and controlled starting seed material. Modifying the standard CTAB protocol by increasing salt concentration in the initial incubation buffer and tightly controlling starting seed material lowers contamination and increases the final concentration of DNA.

[C] DNA migration. The effect excess mucilage has on DNA migration through agarose gels. Lanes 1 and 3; retention of DNA in wells of low percentage agarose gels. Lanes 2 and 4 Selective precipitation of contaminants using NaCl and ethanol results in DNA suitable for electrophoresis.

4.2.2.1 Urea acrylamide gels

Single strand breaks do not disrupt the continuity of the DNA duplex as the structure is maintained by the complementary strand, which also provides the template for repair of the missing nucleotide(s). The repair processes used to repair a SSB depends on how the single strand break forms, but typically they involve four steps: detection, end processing, gap filling and finally ligation (Caldecott, 2008). Under denaturing conditions, the duplex dissociates allowing visualisation of SSBs. Urea is a denaturing agent used to determine DNA single strand breaks using acrylamide gel electrophoresis (section 2.9.3). Polyacrylamide gels are widely used in biochemistry to separate macromolecules such as proteins and nucleic acids based on length and conformation of the molecule. We utilised polyacrylamide gels in an attempt to separate strands of DNA with single strand breaks from intact genomic DNA.

In order to evaluate the sensitivity of this approach to detect and quantify SSBs quantitative, denaturing urea gel analysis was used to analyse standards containing known levels of DNA damage. The rationale behind the approach is that by generating lesion-specific strand breaks utilising glycosylase enzymes with genomic DNA, these can be quantitated using acrylamide gels and image analyses software. Initially synthesised oligonucleotides containing uracil were used. When intact, these oligonucleotides will act as a control where no breaks are present. By treating these oligonucleotides with Uracil-DNA-Glycosylase (UDG), the uracil is removed and single strand breaks are formed. By combining different concentrations of intact DNA and broken DNA we can establish the sensitivity of this method to determine levels of single strand breaks.

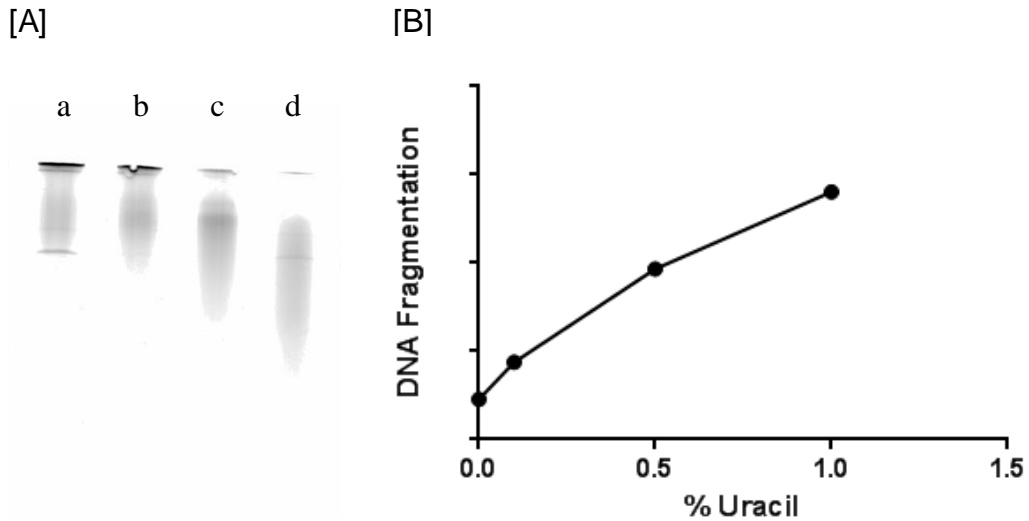


Figure 4.2 The fragmentation of DNA containing differing concentrations of excised uracil molecules

Oligonucleotides containing different percentages of uracil were amplified by PCR and treated with uracil-DNA-glycosylase (UDG) and apurinic/aprimidinic (AP) endonuclease. The resulting products were run on urea acrylamide gels. The shorter, broken, DNA molecules move further down the gel and the amount of DNA migration was calculated.

[A] Urea acrylamide gel with different concentrations of uracil containing DNA. After incubation in UDG, DNA containing uracil in different concentrations were allowed to migrate through the urea acrylamide gel a) 0% Uracil; b) 0.1% Uracil; c) 0.5% Uracil; d) 1% Uracil.

[B] The amount of fragmentation of DNA in a urea acrylamide gel with different uracil concentrations following UDG digestion. DNA containing between 0% and 1% Uracil, were subject to urea conditions and run on acrylamide gels with fragmentation being measured as the amount of DNA migrating away from the intact DNA forming a band towards the top of the gel. As uracil levels increases there is a rise in amount of fragmented DNA passing through the gel.

The same methodology was applied to *Arabidopsis* seeds to quantify the amount of single strand breaks and correlate the data with seed quality to determine any relationship. *Arabidopsis* seed DNA was extracted by chloroform extraction (section 2.6.2) and analysed using urea acrylamide gels as previously described.

When small synthesised nucleotides were run on urea acrylamide gels the separation of DNA containing induced SSBs was detectable at low levels (Figure 4.2). However, similar results were not observed when *Arabidopsis* seed DNA was applied to the acrylamide gels. DNA failed to migrate out of the wells, possibly because of the much greater size of genomic DNA when compared to the synthesised nucleotide or due to an increased mucilage concentration following DNA extraction hindering movement through the crosslinked acrylamide.

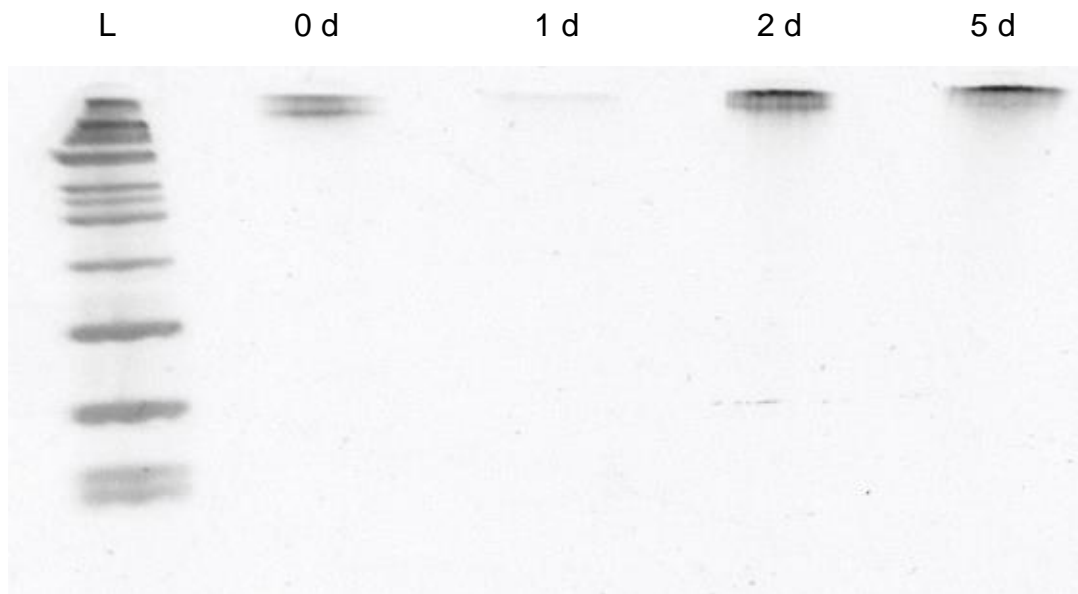


Figure 4.3 Fragmentation of the *Arabidopsis* genomic DNA on urea-acrylamide gels

Extracted DNA from dry *Arabidopsis* seeds was run on urea-acrylamide gels to quantify single strand breaks. (L: bioline hyperladder, 0 d; high quality seeds, 1 d; seeds aged for one day, 2d; seeds aged for two days, 5 d; seeds aged for five days at 35°C, 80% RH)

4.2.2.2 Alkaline agarose gel electrophoresis analysis of DNA from *Arabidopsis* seeds

Alkali is a denaturing agent and, when introduced to agarose gels, allows the separation of DNA molecules up to 20-30 kb, a larger fragment than possible using acrylamide. Alkaline agarose gels were used for analysis of genomic DNA. Initially *Arabidopsis* DNA was extracted from seeds (described in Chapter 3) produced from plants grown under different maternal environments. In addition, some seed lots were subject to ageing treatment as described in Chapter 3. The different conditions during seed development were: low temperature (LT), control temperature (CT) and high temperature (HT) and either left unaged or subjected to accelerated ageing as described in section 3.2.2. The aged samples from each maternal environment were designated as LTA, CTA and HTA respectively. Both LT and CT showed lower viability following ageing with all three conditions showing lower vigour (germination data in section 3.2.2). DNA isolated from these seeds was analysed using agarose gel electrophoresis under alkaline conditions to determine the levels of SSBs in these seeds of differing quality.

Analysis of dry *Arabidopsis* seeds (EcoSeed batches) showed very little loss of integrity (Figure 4.4A.) Neither the maternal temperature or post-harvest ageing conditions used in this study, either singly or in combination, resulted in increased levels of single strand breaks in genomic DNA. The amount of fragmented DNA varied from 0.01% to 0.05%, but significant differences in levels remained difficult to establish because the low levels of fragmentation were near the limit of the sensitivity (detection limit) of this analysis (Figure 4.4B.) However, *Arabidopsis* seeds subject to more severe accelerated ageing regimes (Section 3.1) displayed increased levels of single strand breaks (Figure 4.5). The increase was greatest in seeds after four or five days accelerated ageing (35°C, 80% RH) which lowered germination percentage and vigour significantly (Figure 3.1).

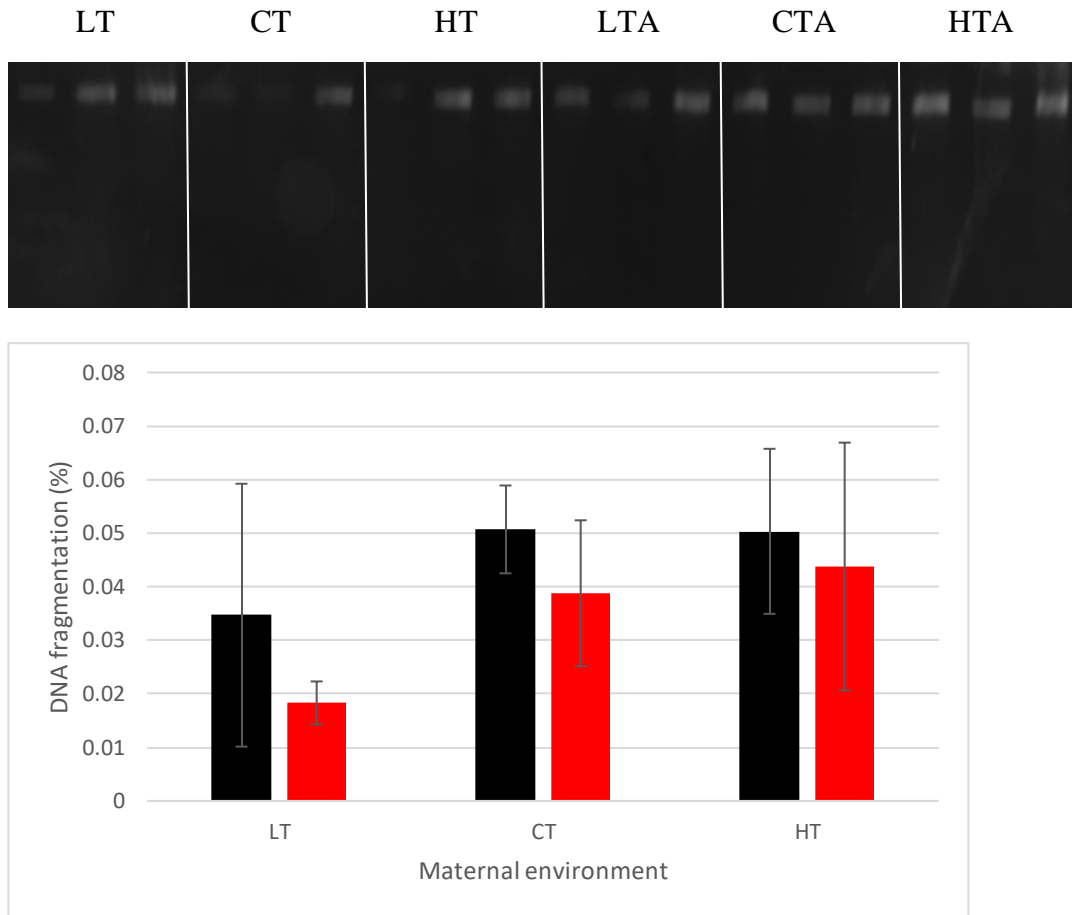
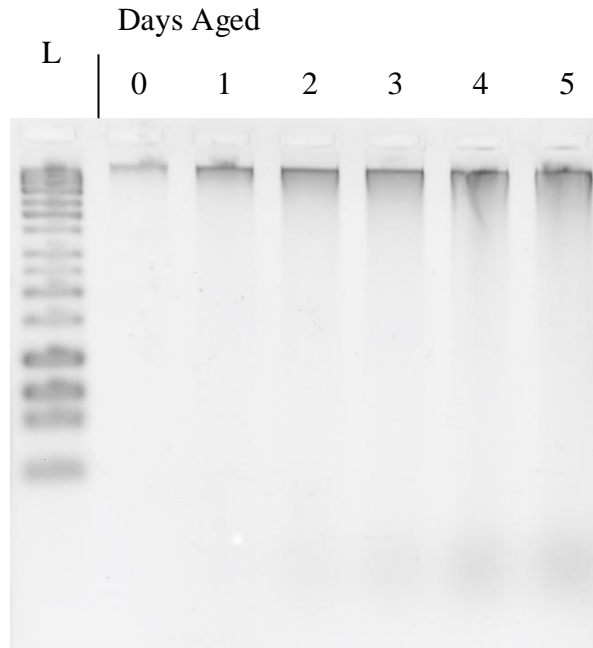


Figure 4.4 Analysis of DNA fragmentation in dry *Arabidopsis* seeds

[A] Alkaline agarose gel analysis of DNA from dry *Arabidopsis* seeds. Intact genomic DNA from dry *Arabidopsis* seeds under different conditions migrates at the top of the gel. Fragmented DNA containing single strand breaks migrates below the intact genomic band. (Maternal environment conditions: **LT**; 14°C-16°C, **CT**; 18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 40°C with 75% relative humidity for 4 days.)

[B] Quantification of fragmented DNA. The percentage of DNA that has migrated further into the gel than the intact genomic DNA. (■) High quality seeds, (■) aged seeds. Error bars denotes \pm SEM (n=3 for each condition).

[A]



[B]

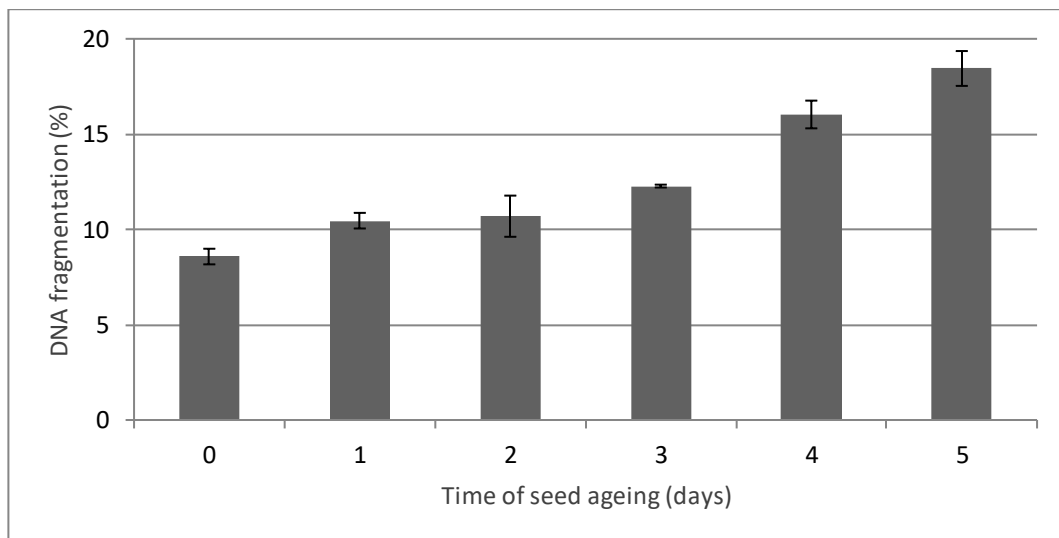


Figure 4.5 Fragmentation of DNA from *Arabidopsis* seed of varying quality

[A] Alkaline agarose gel analysis of DNA isolated from differently aged *Arabidopsis* seeds. Genomic DNA isolated from *Arabidopsis* seeds was analysed by alkaline agarose gel electrophoresis. DNA was detected using GelRed staining and image analysis. L = Bioline hyperladder.

[B] The percentage of DNA that has migrated away from intact genomic DNA. Quantification of fragmented DNA migrating into the gel, expressed as a percentage of total DNA present in the gel. Seeds analysed include high quality (0 days aged)

and seeds aged for periods of 24 hours from 1 day to 5 days (35°C; 75% RH. Error bars represent S.E.M from 3 replicates).

4.2.2.3 Single strand DNA breaks in dry *Brassica* seeds

Levels of SSBs in *Brassica* seeds of differing quality (Chapter 3) were quantified as described above. *Brassica* seeds were produced under control temperature (CT) and high temperature (HT) during formation on the maternal plant. Samples of these seed lots were used to generate aged (A) seed lots using controlled deterioration (CTA; HTA) (40 days; 40°C 75% RH). Seeds produced from plants grown in control conditions showed 3.2% of genomic DNA containing SSBs. Following seed ageing SSB levels in these seed batches rose to 10.1% ($P < 0.05$) of total DNA. A similar effect was seen with the seeds produced at higher maternal environmental temperatures with 3.0% of the DNA containing SSBs in the high quality seeds, rising to 10.3% after accelerated ageing (Figure 4.6; $P < 0.05$).

The differences in maternal environment conditions used in the study did not significantly affect the levels of SSBs present in either the high quality seeds or the seeds that had been aged post-harvest. Seeds from the control maternal conditions showed 3.2% of genomic DNA migrating into the gel, whilst those produced from plants grown in higher temperature conditions showed no difference with 3.0% of fragmented DNA.

4.2.2.4 Accumulation of single strand breaks in imbibed *Brassica* seeds

Brassica seeds were imbibed in water for 10 hours (designated early imbibition time point) or 40 hours (designated late imbibition time point). The early imbibition time point represents the end of phase one of germination where water uptake reaches a stable rate, whilst the late imbibition time point corresponds to 80% of the time required for the first radicle protrusion in control seeds (EcoSeed partner Finch-Savage lab, University of Warwick). This allowed determination of SSB levels throughout seed imbibition, corresponding to DNA repair, or further accumulation of SSBs DNA was extracted from imbibed seeds using the same chloroform based extraction as dry *Brassica* seeds and subjected to alkaline agarose gel electrophoresis.

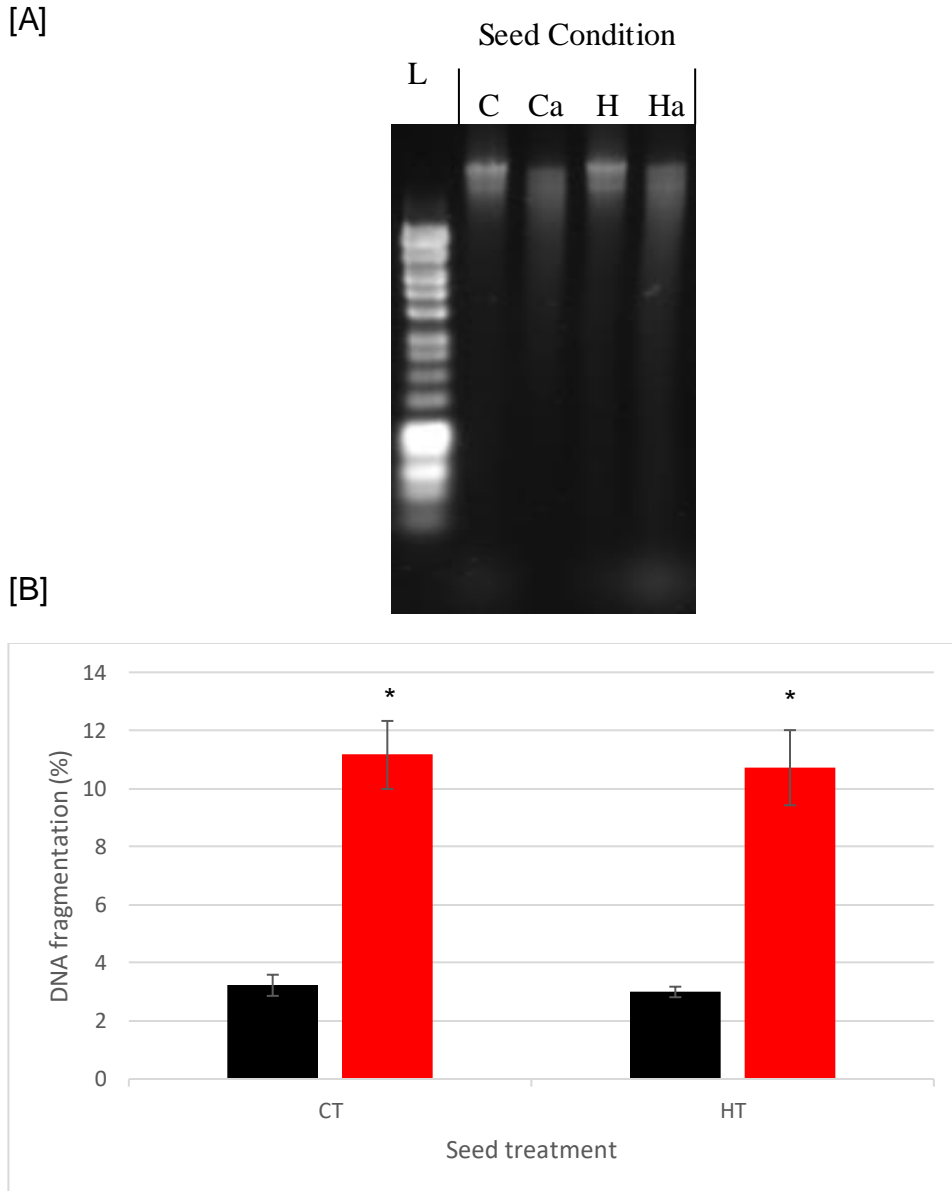


Figure 4.6 Analysis of DNA fragmentation in DNA from dry *Brassica* seeds

[A] Alkaline agarose gel analysis of the DNA of differently aged dry *Brassica* seeds. L; Bioline hyperladder 1 kb, C; Controlled maternal temperature, Ca; controlled maternal temperature and subject to post-shedding ageing, H; high maternal temperature, Ha; high maternal temperature and subject to post-shedding ageing. Full details of conditions are outlined in section 3.2.2

[B] The percentage of DNA that has migrated away from intact genomic DNA. Quantification of the amount of DNA that has migrated further than the intact genomic DNA. (■) High quality seeds, (■) aged seeds. P-values given from Student's T-Test with 3 replicates for each condition, * = $P < 0.05$. Maternal environment conditions: **CT**;

18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 24°C with 75% relative humidity for 10 days and is denoted by the suffix "A" (i.e. CTA and HTA respectively).

Seed ageing resulted in a significant increase in DNA fragmentation in the *Brassica* samples. During early imbibition (10 hours) fragmentation increased in seed harvested from plants grown under control conditions. Perhaps surprisingly, seeds produced under elevated temperatures showed less fragmentation in imbibition, at only 2.48%. DNA fragmentation in imbibed seeds increased significantly following ageing to around 27.87% and 24.47% of the *Brassica* genome respectively (Figure 4.6). At the later stages of imbibition (40 hours) DNA isolated from seeds produced under a controlled maternal environment displayed 2.83% fragmentation and those produced under higher temperature displayed 2.78%. Ageing-induced DNA fragmentation was still apparent at this stage of imbibition, with 20.37% in CT seeds and 30.75% in HT seeds (Figure 4.7).

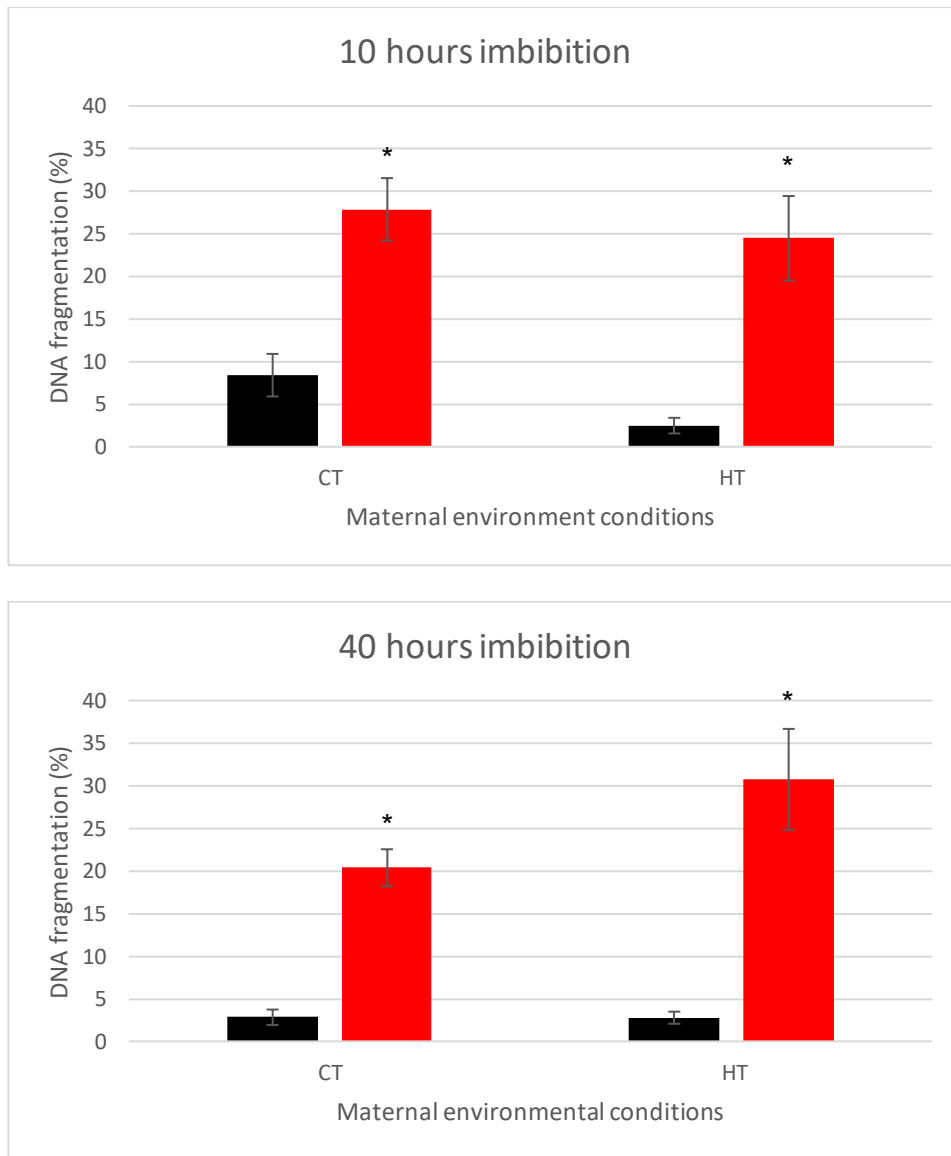


Figure 4.7 Analysis of DNA fragmentation in *Brassica* seeds during imbibition

The percentage of DNA that migrated away from intact genomic DNA in differently treated *Brassica* seeds following imbibition for 10 hours or 40 hours. CT; control temperature during seed formation, HT; high temperature during seed formation. (■) High quality seeds, (■) aged seeds. P-values given from Student's T-Test with 3 replicates for each condition, * = $P < 0.05$. Maternal environment conditions: **CT**; 18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 24°C with 75% relative humidity for 10 days and is denoted by the suffix "A" (i.e. CTA and HTA respectively).

4.3 Discussion

Previous studies identified the accumulation of single strand breaks in aged seeds (Cheah and Osborne). However, this analysis used seed lots with 50% viability, and it remained unclear whether the appearance of SSBs was associated with loss of vigour in viable seeds. In addition, the aim of this study was to determine whether the maternal environment during seed development resulted in changes in seeds produced under these conditions. Low levels of SSBs are tolerated by organisms due to the fact that the physical continuity of the DNA duplex is not disrupted, and the undamaged strand serves as a template for repair. This contrasts with DSBs: due to their high cytotoxicity, even low levels of DSBs can result in cell death. Levels of SSBs can be studied using the Comet assay, single cell electrophoresis in which fragmented DNA migrates as a 'comet' behind the isolated nuclei in an electric field. However, this technique cannot be applied to seeds due to the high levels of background fluorescence in seed samples. Thus denaturing electrophoresis was optimised for analysis of genomic DNA isolated from seeds.

4.3.1 Optimisation of DNA extraction from Arabidopsis seeds

There are several methods of extracting nucleic acids from plant tissue samples. Ensuring there is a robust and reproducible method for extracting high quality DNA from seeds is important in downstream analysis. However different species or lineages, cell or tissue type and presence of potential contaminants dictate which method is optimal (Fang et al., 1992). Seeds are particularly challenging for extraction of DNA. *Arabidopsis* seeds have high levels of seed coat mucilage, storage proteins, lipids and secondary metabolites that makes extraction difficult (Vicient and Delseny, 1999). The removal of undesirable products becomes more difficult through the co-precipitation of mucilage and nucleic acids during the precipitation steps of extraction (Meng and Feldman, 2010). Contaminants can interfere in downstream processes including quantitative methodologies of electrophoresis (this chapter) and PCR-based analysis (Section 6). Carbohydrate contaminants are known to interfere with taq polymerase activity (Fang et al., 1992) and these assays require accurate quantification of genomic DNA used for analysis. Here, a protocol was established to permit

isolation of high quality DNA without co-purifying contaminants. Specifically, optimisation of NaCl, testing concentrations ranging from 0 M to 3.5 M in increments of 0.5 M, determined the ideal concentration for DNA precipitation with no contaminants. In CTAB extraction buffer NaCl prevents DNA from forming precipitates with CTAB, provided it is present in high enough concentrations, and helps increase DNA solubility with reduced levels of fragmentation (Cullings, 1992).

Raising the NaCl concentration alone did not lower contaminant levels sufficiently to obtain high quality DNA from *Arabidopsis* seeds. Several different methods were evaluated to improve DNA purity, including additional washing steps, additional CTAB incubations and different incubation times. By lowering the initial incubation time from 30 minutes at 60°C to two-minute inversion, the yield of DNA recovered was lower but the overall quality increased. Given that DNA is soluble in CTAB extraction buffer, reduction of the incubation period limits the possibility of other molecules co-dissolving. However the main limitation in the yield, and quality, of DNA extracted from seeds (especially after imbibition) was the quantity of starting material used. Upon freezing and grinding seeds in liquid nitrogen, very small amounts were required to extract high quality DNA. In dry seeds the most effective starting weight was 20 mg of ground seed material, which when dissolved in CTAB extraction buffer (6 ml) gives a 0.0012% (w/v) seed content in the solution.

4.3.2 SSB levels in the EcoSeed seed lots

Analysis of seed produced from plants grown under different temperature regimes found no differences in SSB levels. This suggests that growth at these temperatures either did not result in elevated levels of DNA damage in seeds, or that any damage was repaired during the maturation drying phase. There is no direct evidence that growth at lower or higher temperature results in genotoxic stresses. However, temperature stresses can result in elevated ROS levels, either directly or as part of stress signalling (Vickers et al., 2009). However, the growth regimes caused relatively little differences in seed vigour, so it is not surprising that DNA damage was similar between samples. Ageing levels that resulted in changes in seed vigour did lead to significant increases in levels of SSBs. This finding makes a significant advance on the work of

Cheah and Osbourne who demonstrated correlation of low molecular DNA with loss of rye seed viability using alkaline gel electrophoresis (Cheah and Osbourne, 1978). However, this study was flawed, as their seeds showed ~ 50% viability loss. Non-viable seeds may be dead and DNA degradation would occur through the loss of compartmentation and enzymatic degradation in dead tissues. To have biological significance, differences in genome integrity should be detectable between seeds that display a reduction in vigour.

Previous analysis of DNA integrity in pea seeds identified DNA laddering after ageing. This is indicative of programmed nuclease cleavage of DNA into nucleosomal fragments and is a hallmark of programmed cell death (Kranmer et al 2011). In *Arabidopsis* PCD was not detected in alkaline or neutral electrophoresis of genomic DNA.

4.3.3 High levels of single strand breaks accumulate in low quality *Brassica* seeds

Here, ageing showed a negative effect on DNA integrity with a significant rise in single strand break presence in all *Brassica* seed lots tested following accelerated ageing (Figures 4.6 and 4.7). The amount of SSBs in seeds grown in higher temperatures did not differ significantly from those grown in control temperature. Upon imbibition *Brassica* seeds accumulated single strand breaks at a higher rate than when desiccated. Both unaged and aged seeds grown in control temperatures saw frequencies of SSBs rise ~2 fold, with a similar rise seen in aged seeds grown at a higher temperature. There was no change in unaged seeds grown at a higher temperature following imbibition. Looking specifically at aged seeds, those grown in control temperature dropped slightly, but not significantly, between early and late imbibition time points ($P>0.05$). However seeds grown at a higher temperature and subject to ageing saw levels of SSB continue to rise from 24.5% to 30.7%. The increase in single strand break presence correlates with the loss of vigour seen in *Brassica* seeds (section 3.2.3). The instances of single strand breaks, especially at late germination, show the same trend as viability and vigour differences. HTA seeds have the poorest germination performance, reducing in viability to 56.7% and these seeds show the highest level of fragmentation.

Conversely HT seeds germinate quickest and have the highest viability and also have the lowest SSB presence during late imbibition (Figure 4.7).

The rate of base loss in dry seeds is reduced due to their desiccated state (Dandoy et al., 1987), potentially accounting for the low rates of loss in *Arabidopsis*, and although there were still large differences in dry *Brassica* seeds the levels rose significantly during imbibition. This correlates to work undertaken in corn plants (*Zea mays*) where within the first 20 hours of imbibition AP sites rose 4-fold, as the suspected action of DNA glycosylases activation, following initiation of respiration (Dandoy et al., 1987). Although the corn seeds were stored for 2 years at 20°C DNA damage is still evident in seeds of high quality due to the inherent instability of DNA in the cellular environment (Lindahl, 1993). This is also seen in both *Arabidopsis* and *Brassica* dry seeds (Figures 4.5 and 4.6) where, although high quality seeds have fewer DNA strand breaks, they are still present. This contrasts with the *Arabidopsis* seed lots, which showed no significant difference in SSB levels under the ageing conditions employed by EcoSeed (Figure 4.4) and SSBs only became apparent at higher levels of ageing (Figure 4.5). Thus, neither different maternal environments, nor post-harvest ageing of the EcoSeed *Arabidopsis* seed lots led to detectable differences in DNA fragmentation, despite the lower vigour and viability of the seed lots (Figure 3.2).

Brassica seeds contain higher levels of hydrogen peroxide (H_2O_2) and lower levels of total glutathione (GSH) following ageing (EcoSeed partner; Kranner lab, University of Innsbruck). Cysteine, an important molecule in GSH metabolism, also increases to almost three-fold normal levels following accelerated ageing of *Brassica* seeds. Considering the importance of protecting the genome from oxidative damage, the lower levels of GSH, an important non-enzymatic antioxidant, could account for a decreased ability to protect the genome from reactive oxygen species (ROS) in aged seeds, causing increase single strand breaks as a result of repair processes (Section 1.2.2.2.). Furthermore higher levels of H_2O_2 in aged seeds could correspond to an increased levels, in DNA damage as several cell types have shown induced single and double strand breaks in the presence of hydrogen peroxide (Ananthaswamy and Eisenstark, 1977; Olson, 1988; Driessens et al., 2009).

GSH oxidative protection becomes more pertinent in the dry seed due to low levels of metabolism hindering enzymatic damage prevention from enzymes such as superoxide dismutases (SODs) and catalase (Bewley, 1997). Ageing has been shown to increase oxidation of molecules within the dry seed such as proteins, DNA and lipids (Bailly, 2004; Job et al., 2005; Waterworth et al., 2016).

4.3.4 The effects of SSBs in seeds

Single strand breaks are less cytotoxic and recombinagenic than DSBs as the duplex remains intact and the complimentary DNA strand provides a template for SSB repair. However, single strand DNA breaks are potentially severe lesions and occur at much greater frequency than double strand breaks. During DNA replication or transcription, helicases separates the two strands of the duplex decreasing overall stability. During replication if a polymerase encounters a single strand break there is the potential for termination and generation of a double strand break, creating loss of genetic content (Mannuss et al., 2010; Waterworth et al., 2011). Double strand breaks are generally considered the most potent form of DNA damage because of the propensity for loss of genetic information leading to genotoxic or cytotoxic events. Genomic instability accumulates in seeds with reduced viability, with reduced quality seeds showing chromosomal abnormalities. Lettuce (*Lactuca sativa*) seeds showed 90% abnormal anaphase in seeds with a 50% loss in viability indicating the importance for genome integrity in high quality seeds (Villiers, 1974). Cytogenetic analyses identified that aged *Arabidopsis* seeds showing delayed germination but no loss of viability carried anaphase bridges (chromosomal fusions) in 4% cells of germinating embryos.

Single strand breaks are also intermediates formed during the regular cellular repair of base adducts, one of the most common forms of lesions. The removal of apurinic/apyrimidinic sites, as mediated by the enzyme ARP (apurinic endonucleonuclease-redox protein) generates single strand breaks and is the most active glycosylase in *Arabidopsis* during imbibition (Córdoba-Cañero et al., 2011). Thus, the combination of SSBs incurred through imbibition and as a result of repair processes initiated on the resumption of metabolism could generate high levels of single strand breaks. Here we identified the

accumulation of these lesions during imbibition of *Arabidopsis* and *Brassica* and found increased levels of SSBs in aged seeds associated with loss of vigour.

5. Cellular responses to DNA damage in seeds

5.1 Introduction

All organisms encounter stress-induced genomic damage which can cause mutations and cell death. Genomic integrity is of prime importance for survival and transfer of genetic information to future generations. Several DNA repair pathways and DNA damage specific responses have evolved to minimise the harmful effects of the DNA damage to the cell and the organism and to aid in the repair of lesions before cell cycle progression results in permanent mutation of the genome.

Plants have developed powerful responses to DNA lesions collectively termed the DNA damage response (DDR) pathway. One pathway particularly important to the cellular response to DNA double strand breaks is mediated by the checkpoint kinases ATAXIA TELANGIECTASIA MUTATED (ATM), with additional signalling activity also mediated by the related kinase ATAXIA TELANGIECTASIA-MUTATED and Rad3-related (ATR). Double strand breaks are the most cytotoxic form of DNA damage with single DSB capable of causing cell death in yeast (Bennett et al., 1996). ATM is essential in coordinating downstream processes following DSB formation in order to limit further damage and initiate repair (Garcia et al., 2003). ATR on the other hand responds more strongly to replication defects and is activated by single stranded regions of DNA which are formed as a result of replication stress (Culligan et al., 2006).

There are several processes initiated by the DDR in plants which are important for survival. These processes are also observed in seeds during the early stages of imbibition, where the influx of water and resumption of metabolic activity is accompanied by detection of damage accumulated during quiescence (Dandoy et al., 1987). DNA damage levels are elevated in aged seeds, resulting in differences in vigour and viability (Cheah and Osborne, 1978; Waterworth et al., 2010). Several ATM mediated responses activate early in imbibition to combat DSBs including a transcriptional upregulation of DNA repair associated genes, phosphorylation of histones and activation of cell cycle checkpoints (Falck et al., 2005; Waterworth et al., 2010; Waterworth et al., 2016).

Within the first 6 hours of imbibition in *Arabidopsis* there is a large increase in transcripts of DNA repair factors including RAD51, RNR, TK, PARP and WEE1. RADIATION SENSITIVE 51 (RAD51) is involved in homology searching during homologous recombination, one of the major pathways of DNA double strand break repair. Permanent activation of *RAD51* is seen in mutant plants with genome instability, including DSB repair mutants (West, 2004) and plants defective in chromatin assembly (Endo et al., 2006). RIBONUCLEOTIDE REDUCTASE (RNR) and THYMIDINE KINASE (TK) are both important in the generation of new deoxynucleotides for incorporation in repair synthesis of damaged DNA. Both genes are upregulated in the presence of increasing double stranded DNA damage and *RNR* is also transcriptionally induced in times of replication stress (Chen et al., 2010; Jossen and Bermejo, 2013). POLY(ADP-RIBOSE) POLYMERASE (PARP) catalyses the addition of poly(ADP)ribose to proteins in response to DNA DSBs, signalling and recruiting further repair proteins to sites of genomic damage (Beck et al., 2014). WEE1 negatively affects the action of CYCLIN DEPENDENT KINASE A;1 (CDKA;1), especially in the presence of double strand breaks, causing cell division arrest (Ricaud et al., 2007). All the genes listed above show increased transcriptional activity in the presence of double strand breaks, as mediated by the phosphatidylinositol protein kinase like kinase (PIKK) ATM (Culligan et al., 2006). Several also show increased activity early in imbibition of *Arabidopsis* seeds (Waterworth et al., 2010). The presence of these transcripts throughout imbibition therefore should give an indication of the detection during imbibition of DNA double strand breaks accumulated during storage.

5.1.1 Aims

Previous analysis identified transcriptional induction of the DDR within the first few hours of imbibition in response to DNA damage incurred during storage of the quiescent seed. This response is indicative of the induction of repair processes early in imbibition. This response is evidenced even in freshly harvested high quality *Arabidopsis* seeds (Waterworth et al., 2010), and less is known on how this response would be affected in low-quality seeds with an

increased amount of DNA damage present (Abdalla and Roberts, 1969; Weimer et al., 1972; Cheah and Osborne, 1978; Osborne et al., 1984).

Through measuring transcript levels of the DNA repair-associated proteins involved in the DDR we can determine whether increased DNA damage associated with seed ageing results in a stronger response and a higher accumulation of these repair transcripts. These transcriptional changes are readily quantifiable and therefore have potential use as markers for seed quality. Transcript levels were therefore quantified using PCR-based approaches. Furthermore, tissue specificity of expression is determined using GUS-reporter constructs linked to the promoters of selected DDR genes. Histochemical approaches allow cell and tissue specific expression patterns of gene expression to be identified which can provide insight into the physiological functions of repair gene expression in seeds. The changes in transcript levels of four genes were determined: *RAD51*, *TK1*, *PARP2* and *RNR*. These genes were selected as they showed the highest induction, following imbibition, of DNA repair-related genes. In order to account for normal transcriptional differences upon imbibition, all genes were standardised to *ACTIN7*. *ACT7* displays no differences in transcript level dependent on seed condition or ageing regime (Rajjou et al., 2008).

5.2 Results

5.2.1 The transcriptional response in *Arabidopsis*

5.2.1.1 Transcriptional induction of *RAD51* during imbibition after maternal and post-harvest stress

RADIATION SENSITIVE 51 (*RAD51*) is protein essential for homologous recombination (HR) repair of double stranded DNA breaks and *rad51* knockout mutants are sterile in *Arabidopsis* due to the failure to repair meiotic DSBs (Li et al., 2004). Levels of *RAD51* transcript have been shown to increase in the early hours of imbibition as part of the DNA damage response (DDR) (Waterworth et al., 2010). Here we looked at the difference in transcript levels at three time-points: dry seed, 6 hours imbibition (early imbibition; EI), and 72 hours imbibition (late imbibition; LI) following temperature stress on the maternal plant and post-harvest ageing.

Levels of *RAD51* transcripts were measured and normalised to *ACTIN7* in seeds of different quality across imbibition. Low levels of transcripts were seen in dry seeds and there was no difference between high quality seeds grown in the control temperature and any other maternal or post-harvest ageing conditions before imbibition. Transcript levels increased following imbibition for 6 hours in all maternal conditions that were not subjected to accelerated ageing (LT, CT, HT) in comparison to control temperature dry seeds. Seeds grown in low temperatures had a 13.8-fold increase in *RAD51* transcript levels; however this was not seen in the same seeds that had undergone accelerated ageing (4.9-fold increase). Similar trends were seen in seeds at control temperature and high temperature at early imbibition. Levels of transcripts in CT seeds increased 11.8-fold compared to only a 1.4-fold increase in the same seeds following ageing. HT seed *RAD51* transcript levels increased 10.1-fold whereas HTA seed transcripts only displayed a 3.3-fold increase. All high quality seeds (those that had not gone post-harvest accelerated ageing) displayed increased levels of *RAD51* following 6 hours imbibition with transcript levels reducing by the 72 hour time-point. There was no increase in the DDR response to seed ageing treatments (Figure 5.1). This suggests that

there is reduced capacity for aged seeds to mount the transcriptional DDR upon imbibition.

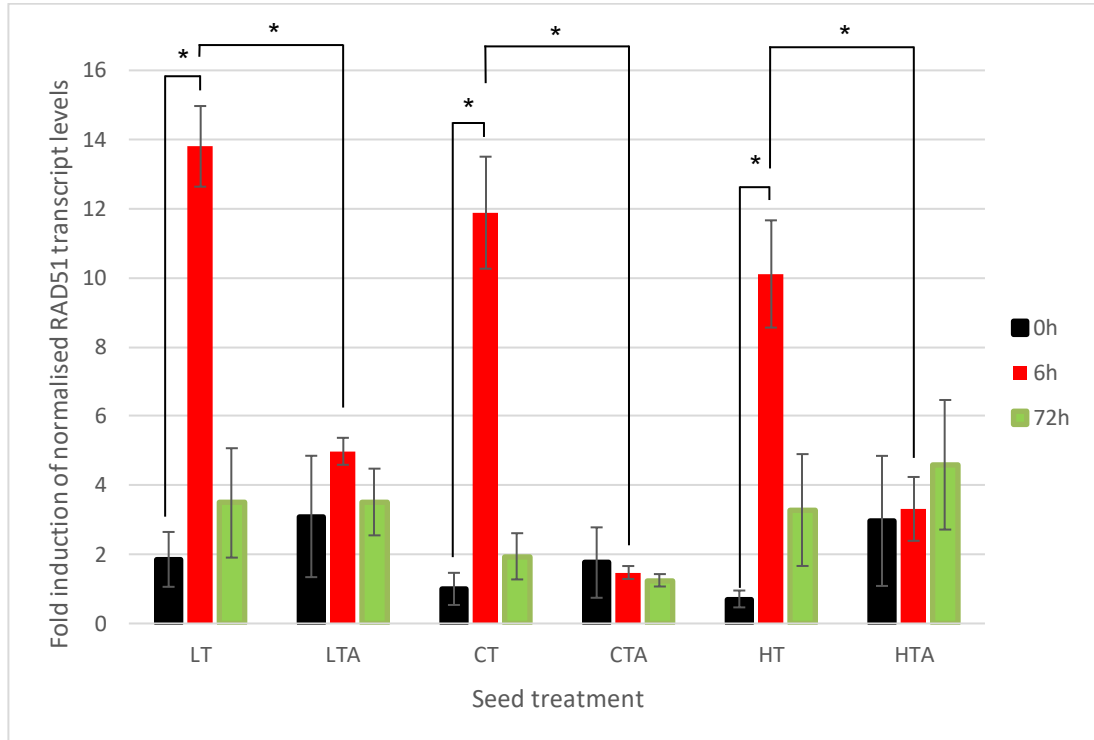


Figure 5.1 *RAD51* transcript levels in seeds during imbibition

Levels of *RAD51* transcripts were measured in dry seeds (■), seeds at an early imbibition time of 6 hours (■) and seeds at a late imbibition time of 72 hours (■). The seeds were of different quality after undergoing temperature stresses whilst developing on the mother plant along with post-harvest ageing (see section 3.2.2 for full seed treatment notations). All values are standardised to levels found in dry seed grown at a control temperature and without any accelerated ageing (CT). There were rises in transcript levels in early imbibition (6 hours) in all seeds that had not undergone accelerated ageing and those seeds that were aged showed an inability to initiate the same increases in *RAD51* transcript levels ($P < 0.05$; Student's T-Test). Levels of *RAD51* were normalised to *ACTIN7* and expressed as fold-change from transcript levels in the unimbibed seed. P-values given from Student's T-Test with 3 replicates for each condition, * = $P < 0.05$. Maternal environment conditions: **LT**; 14°C-16°C, **CT**; 18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 40°C with 75% relative humidity for 4 days and denoted as LTA, CTA and HTA respectively.

5.2.1.2 Transcriptional induction of *RNR* during imbibition after maternal and post-harvest stress

RIBONUCLEOTIDE REDUCTASE (*RNR*) is an enzyme involved in the synthesis of deoxyribonucleotides from ribonucleotides and has important roles in DNA synthesis and repair (Herrick and Sclavi, 2007). *RNR* is one of many DNA repair related transcripts shown to increase in abundance following imbibition. Here we looked to determine whether different maternal plant environments affected the *RNR* transcriptional response upon imbibition. We also looked at levels of *RNR* transcripts in quiescent and imbibition seeds following post-harvest ageing to determine whether the accumulation of DNA damage associated with ageing affected the *RNR* transcriptional response.

Transcript levels of *RNR* (normalised to *ACTIN7* levels in dry seed) were measured seeds imbibed for 6 hours (early imbibition time-point) and seeds imbibed for 72 hours (late imbibition time-point). *RNR* transcript levels increased following 72 hours imbibition in all unaged samples (LT, CT, HT) along with CTA seeds (seeds subjected to accelerated ageing following formation in controlled maternal environmental temperature) compared to high quality, dry CT seeds ($P < 0.05$). The two remaining aged samples at suboptimal temperatures (LTA, HTA) showed no significant change in *RNR* transcript levels. At the late imbibition time point transcript levels in seeds grown at low (LT) and control (CT) temperatures were higher than in the same seeds that had undergone accelerated ageing (LTA and CTA respectively) ($P < 0.05$). *RNR* transcript levels did not change at the early imbibition time-point in any of the conditions tested suggesting *RNR* is not actively transcribed until after 6 hours in the conditions used here (Figure 5.2).

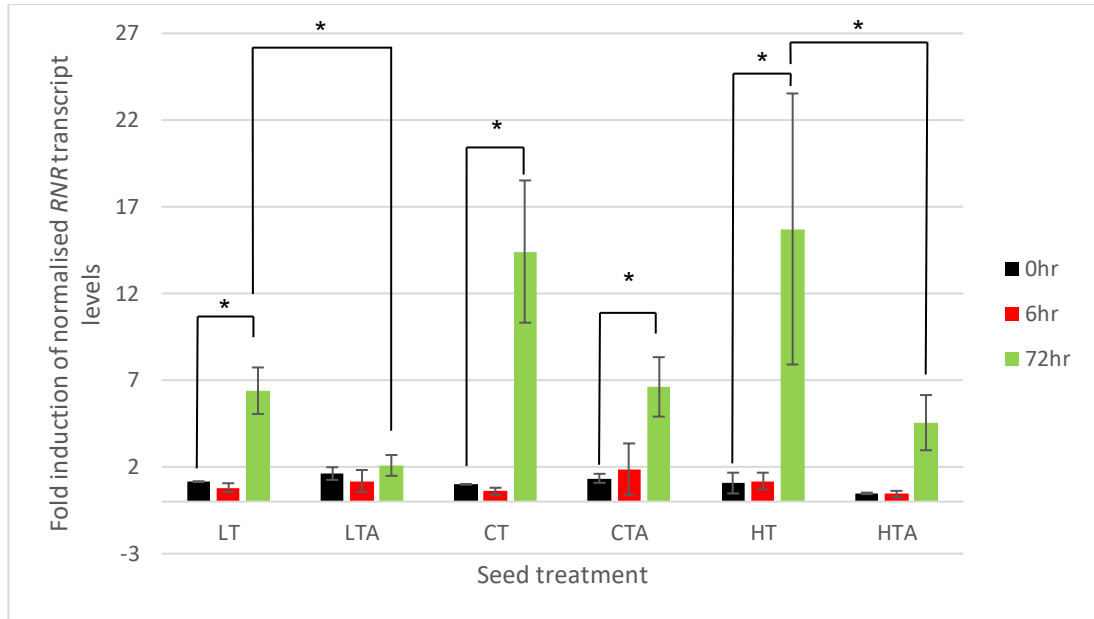


Figure 5.2 *RNR* transcript levels in seeds during imbibition

Levels of *RNR* transcripts were measured in dry seeds (■), seeds at an early imbibition time of 6 hours (■) and seeds at a late imbibition time of 72 hours (■). The seeds were of different quality after undergoing temperature stresses whilst developing on the mother plant along with post-harvest ageing (see section 3.2.2 for full seed treatment notations). All values are standardised to levels found in dry seeds grown at a control temperature and without any accelerated ageing (CT). There were rises in transcript levels in late imbibition (72 hours) in all seeds that had not undergone accelerated ageing, along with seeds that had been aged and kept in a control-temperature maternal environment. The remaining aged seeds (LTA, HTA) showed an inability to initiate the same increases in *RNR* transcript levels following imbibition ($P < 0.05$; Student's T-Test). *RNR* transcript levels were normalised to *ACTIN7* and expressed as fold-change from transcript levels in the unimbibed seed. P-values given from Student's T-Test with 3 replicates for each condition, * = $P < 0.05$. Maternal environment conditions: **LT**; 14°C-16°C, **CT**; 18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 40°C with 75% relative humidity for 4 days and denoted as LTA, CTA and HTA respectively.

5.2.1.3 Transcriptional induction of *THYMIDINE KINASE (TK)* during imbibition after maternal and post-harvest stress

Two orthologues of THYMIDINE KINASE (TK) are present in *Arabidopsis*: TK1a and TK1b and show redundancy (Pedroza-Garcia et al., 2015). TK is found in most living cells and catalyses the reaction of thymidine into thymidine monophosphate, one of the four main nucleotides in the plant nuclear genome. This reaction is important in the synthesis of thymidine triphosphate and subsequent incorporation of thymidine into the genome during DNA repair synthesis and DNA replication. We investigated the expression of this gene by quantifying *TK* transcripts in dry seeds and at early and late imbibition time points, normalised to *ACTIN7*. *RAD51* and *RNR* displayed a reduction in imbibition-induced transcript level increases following post-harvest ageing, this same trend was not evident with *TK*. After six hours imbibition there was a reduction in transcript levels in aged seeds produced under low temperature conditions (LTA) in comparison to the same seeds of higher quality (LT) ($P < 0.01$). The expression profile suggests thymidine kinase transcript levels are similar to *RNR*, with levels rising in late imbibition (Figure 5.3).

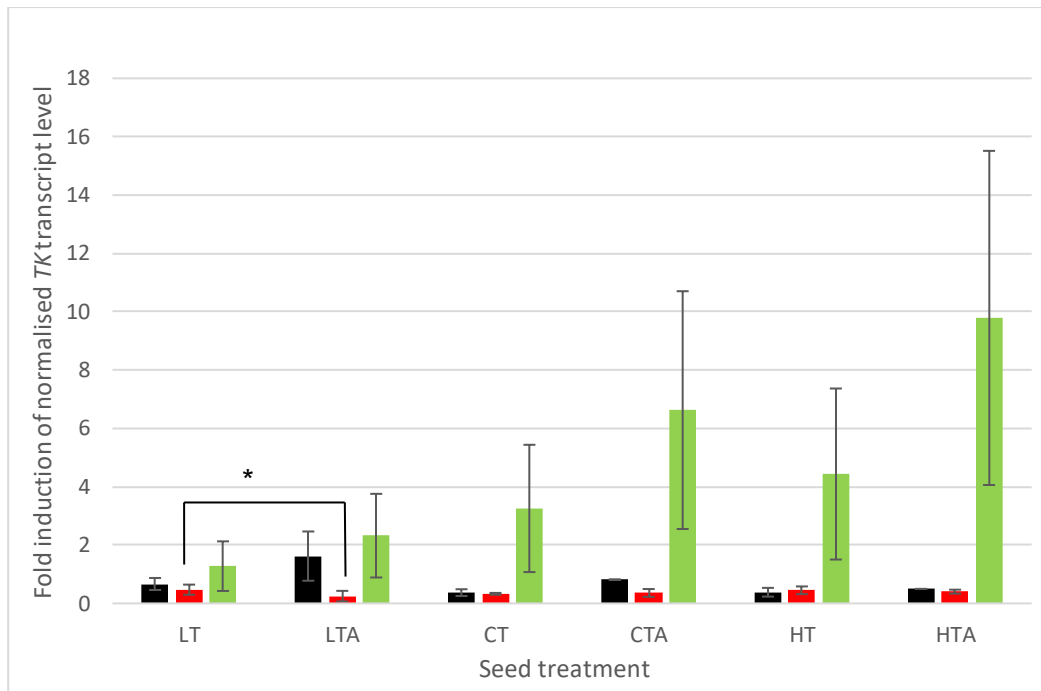


Figure 5.3 *TK* transcript levels in seeds during imbibition

Levels of thymidine kinase transcripts were measured in dry seeds (■), seeds at an early imbibition time of 6 hours (■) and seeds at a late imbibition time of 72 hours (■). The seeds were of different quality after undergoing temperature stresses whilst developing on the mother plant along with post-harvest ageing (see section 3.2.2 for full seed treatment notations). All values are standardised to levels found in dry seeds grown at a control temperature and without any accelerated ageing (CT). Unlike the transcript levels of *RAD51* and *RNR* there was no overall trend seen with the different conditions except between LT and LTA where thymidine kinase transcript levels were lower following post-harvest ageing following 6 hours imbibition ($P < 0.01$; Student's T-Test). *TK* transcript levels were normalised to *ACTIN7* and expressed as fold-change from transcript levels in the unimbibed seed. P-values given from Student's T-Test with 3 replicates for each condition, * = $P < 0.05$. Maternal environment conditions: **LT**; 14°C-16°C, **CT**; 18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 40°C with 75% relative humidity for 4 days and denoted as LTA, CTA and HTA respectively.

5.2.1.4 Transcriptional induction of *POLY(ADP-RIBOSE) POLYMERASE (PARP)* during imbibition after maternal and post-harvest stress

POLY(ADP-RIBOSE) POLYMERASE (PARP) is a family of proteins that mediates the transfer of ADP-ribose groups to proteins in response to genotoxic stress (Waterworth et al., 2015). PARP proteins have been shown to be important in germination and activity has been strongly implicated in the presence of strand breaks (Hunt et al., 2007; Briggs and Bent, 2011). There are three major PARP proteins in *Arabidopsis*: PARP1, PARP2 and PARP3. PARP1 accounts for over 90% of DNA damage-related PARP activity in humans but PARP2 is responsible for the majority of repair in *Arabidopsis* (Song et al., 2015). PARP3 has roles in the storability of seed, with *parp3* mutants showing increased sensitivity to ageing and lower seed viability (Rissel et al., 2014). *PARP2* transcript levels are induced as part of the DNA damage response in seeds (Waterworth et al, 2010) and in the current study *PARP2* levels were measured throughout imbibition in *Arabidopsis* seeds of varying quality.

The levels of *PARP2* transcripts were lowest in dry seeds with little change regardless of ageing regime or maternal environment as with the previous DNA-repair related transcripts. *PARP2* levels increased following 72 hours imbibition in CTA ($P < 0.05$), HT and HTA seeds ($P < 0.01$; Figure 5.4) but not in seeds produced at lower temperatures or the unaged control temperature samples. There was no difference between any of the high quality seed batches following post-harvest ageing and similarly no significant difference was seen between differing environments during seed maturation.

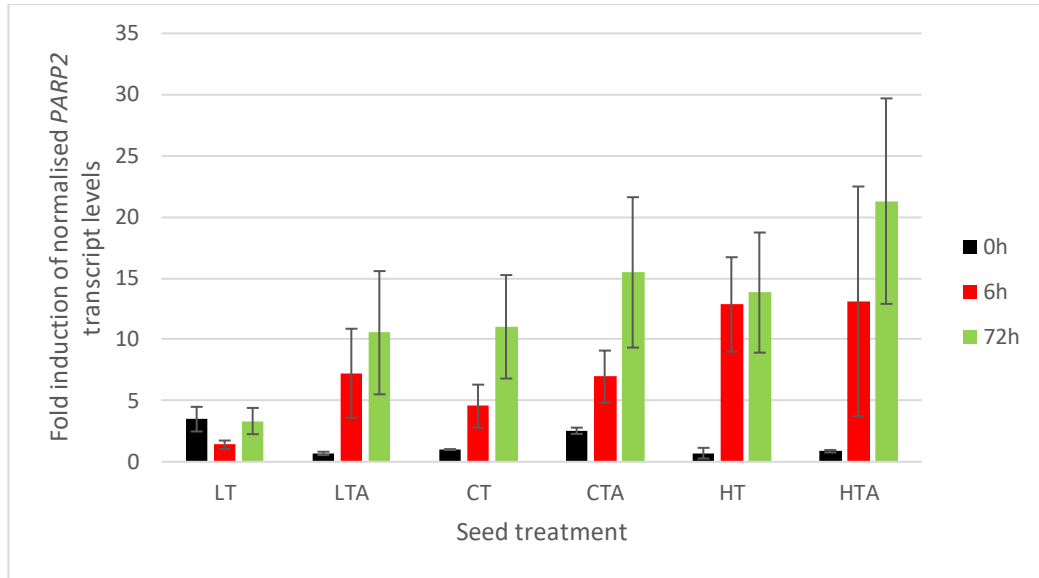


Figure 5.4 *PARP2* transcript levels in seeds during imbibition

Levels of *PARP2* transcripts were measured in dry seeds (■), seeds at an early imbibition time of 6 hours (■) and seeds at a late imbibition time of 72 hours (■). The seeds were of different quality after undergoing temperature stresses whilst developing on the mother plant along with post-harvest ageing (see section 3.2.2 for full seed treatment notations). All values are standardised to levels found in dry seeds grown at a control temperature and without any accelerated ageing (CT). Ageing generated no significant differences in transcript abundance, nor did maternal environmental differences. 3 replicates were used for each condition with no significant difference across treatments. Maternal environment conditions: **LT**; 14°C-16°C, **CT**; 18°C-22°C, **HT**; 25°C-28°C. Controlled deterioration of seeds undertaken at 40°C with 75% relative humidity for 4 days and denoted as LTA, CTA and HTA respectively.

5.2.1.5 Modification of the transcriptional response in seeds of lower quality

The previous sections indicate that seed ageing modifies the transcriptional response, resulting in a lower level of induction of certain genes under the control of the DNA damage response pathway. Aged seeds that had been imbibed for 6 hours were tested to determine whether the severe accelerated ageing regimes reduces the transcriptional induction seen upon rehydration. The increase in normalised transcript levels at 6h imbibition relative to levels in the dry seed (0 hour time point) were compared between unaged seeds and seeds that had been aged for 3 days as described in section (section 3.2.1) (Figure 5.4).

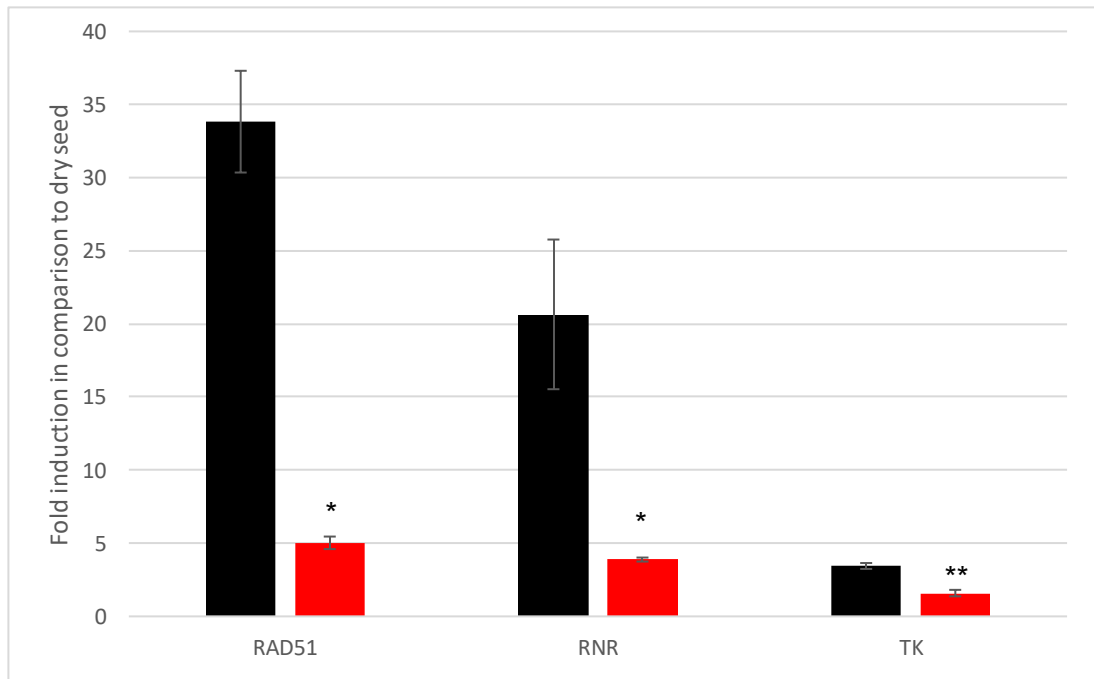


Figure 5.5 *RAD51*, *RNR* and *TK* transcript levels following accelerated ageing

Levels of *RAD51*, *RNR* and *TK* transcripts were measured in high quality *Arabidopsis* seeds (■) and seeds that had undergone accelerated ageing for 3 days (■) after 6 hours of imbibition. All values are given as a fold increase from dry seed of the respective treatments. Levels of transcripts were lower in aged seeds compared to the high quality seeds for all three genes (*: $P < 0.05$; **: $P < 0.01$. Student's T-Test, $n = 3$ batches of seeds per condition). Accelerated ageing was undertaken at 40°C and 75% RH.

High quality seeds had higher levels of transcripts of all three DDR genes analysed. *RAD51* transcripts were the highest of the three genes following 6 hours of imbibition with an increase of 35.5-fold. Following accelerated ageing, the transcriptional response was reduced to only a 4.2-fold increase in *RAD51* levels. Similar effects were observed in *RNR* transcript levels, where high quality seed levels increased to 20.6-fold, and ageing again caused a reduction in transcriptional response, at only 3.9-fold levels observed in dry seeds. *TK* showed a lower induction in comparison to the other genes, with high quality seeds showing a 3.4-fold increase in transcript levels upon imbibition. Despite the relatively lower induction of *TK* compared to the other genes, subjecting the seeds to accelerated ageing still reduced transcriptional induction of *TK* with those seeds showing a 1.6-fold increase (Figure 5.3). These results suggest that ageing impairs the transcriptional response, rather than any increased DNA damage producing stronger DNA damage responses on imbibition.

5.2.2 Temporal and spatial localisation of DNA repair genes

The abundance of transcripts associated with the DDR changes throughout imbibition, possibly depending on the requirement for repair and the ability of the seed to mount an adequate response. Increasing understanding of the plant DDR will determine the differentiation of DDR activation between cell types. All cells in plants arise from the root apical meristem (RAM) and the shoot apical meristem (SAM); consequently, maintenance of genomic integrity is of prime importance in meristem cell where mutations will perpetuate in the plant germline. Cell division in germination is initiated in the RAM and SAM around the time of germination (Masubelele et al., 2005). Recent studies identify the critical roles ATM plays in delaying progression of germination in aged seeds to safeguard the genome from high levels of genotoxic stress sustained in desiccation and quiescence. ATM functions through transcriptional induction of the cell cycle inhibitor SMR5 in the RAM (Waterworth et al., 2016). One possibility could be that the DDR is elevated or localised to the RAM or SAM.

Therefore, it is plausible the DDR might be restricted or enhanced in certain cell types, whilst different component transcripts of the plant DDR might show different spatial and temporal patterns of expression.

Analysis of transcript levels in the whole seed does not provide insight into the cell specific localisation of the DDR associated genes in *Arabidopsis* seed. The change in transcript levels allowed for determination of temporal changes in mRNA abundance of repair genes by using RT-PCR in the previous sections. Whereas this establishes the total levels of transcripts at different points throughout imbibition, here we employed GUS-reporter studies to investigate spatial changes in gene expression. By using GUS-promotor fusions of DDR associated genes we determined the cellular specific expression pattern and thus where repair activities are most needed.

The GUS reporter gene was fused to the promotor region of repair genes, in this case PARP, RNR, TK and WEE1. *WEE1:GUS* lines were kindly supplied by van Lieven de Veylder (Ghent University) in *Arabidopsis* Col-0. Other lines were generated in pCB1381z and introduced into Col-0 lines using *Agrobacterium* mediated transformation (section 2.10). Homozygous transformants were isolated and the GUS-expressing seeds are collected and, following after-ripening, are kept as high quality standards or aged for three days as described in (section 2.5.3; 35°C, 80% RH). Expression patterns during imbibition can then be analysed using histochemical staining.

Four time points were selected to relate to different physiological stages of seed imbibition: 1) dry seed, 2) 6 hours imbibition (to represent early imbibition when metabolism has begun and transcriptional DDR peaks (Waterworth et al., 2010)), 3) seed cracking and 4) germination. Germination is defined as the point in which the radicle emerges from the seed coat. Seed cracking precedes this point, marked by seed coat ruptures but elongation has not yet occurred. Previous studies identify that cell cycle activation occurs around the time of seed coat rupture, with initiation of DNA replication (Waterworth et al., 2016). The last two time points were selected to establish expression of DDR genes around the time of cell cycle initiation to relate this to seed quality (Barrôco et al., 2005; Masubelele et al., 2005; Waterworth et al., 2016). By choosing the stages in development rather than set time-points it allows us to look at high

quality seeds and aged seeds in terms of physiological changes at important stages of imbibition, even though both sets of seeds will reach these stages at different times.

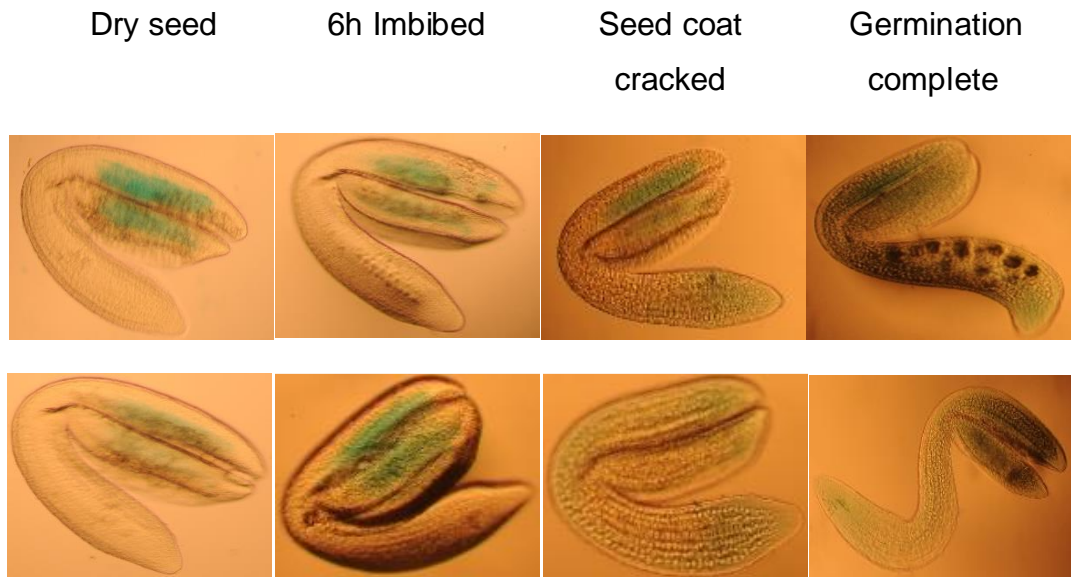


Figure 5.6 PARP:GUS localisation throughout imbibition

Representative images showing the pattern of PARP2:GUS expression was analysed by PARP:GUS localisation in high quality seeds (top row) and in seeds following accelerated ageing (bottom row) at the following stages: dry seeds, 6h imbibed seeds, seeds following coat cracking and after germination. A minimum of 15 seeds were isolated per condition.

Analysis of PARP:GUS enzyme activity throughout imbibition identified several changes in expression. Firstly, when comparing the differences between high quality (top row) and low quality (bottom row) dry seeds (Figure 5.5A) it appears that PARP is expressed in dry seeds, localised within the cotyledons, presumably carried over from the late stages of maturation drying. Furthermore, levels in aged seeds were lower than that in high quality seeds, possibly reflecting GUS inactivation in ageing. The localisation of PARP:GUS within the cotyledons remains similar following imbibition for 6 hours; however the intensity of staining in aged seeds increases whereas high quality seeds show a slight reduction. As the seed coat ruptures almost all *PARP:GUS* transcription stops within the cotyledons, with some detectable levels in the high quality seeds. Localised *PARP* expression is observed at root tip where cell cycle will be initiated in the RAM (Masubelele et al., 2005). After germination (Figure 5.5D) low levels were observed distributed throughout the entire aged embryo, whilst high quality seedling had more concentrated levels in the root tip and in the cotyledons, consistent with elevated repair level in active meristems (Yadav et al., 2009).

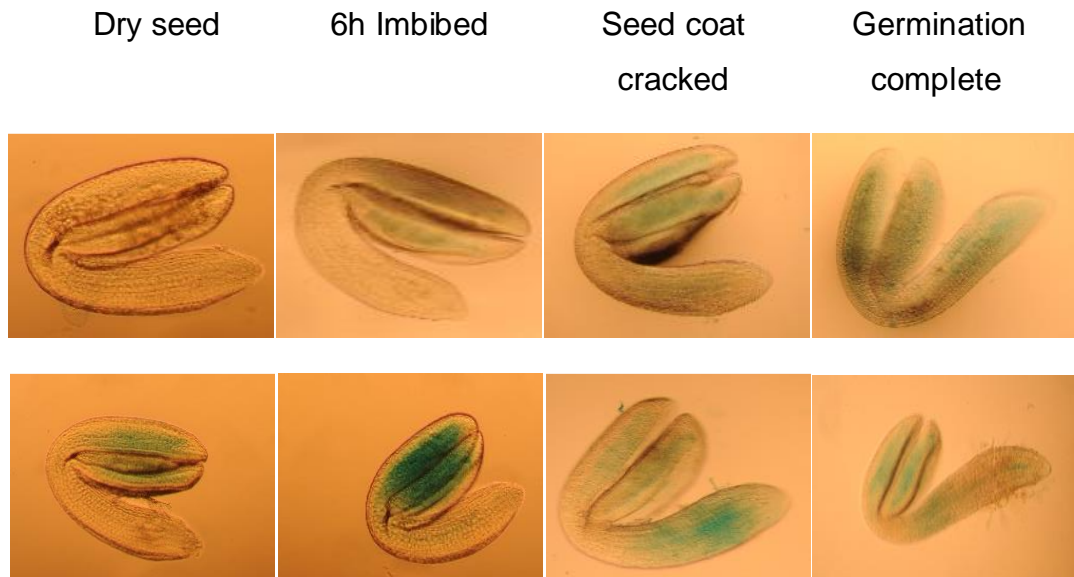


Figure 5.7 RNR:GUS localisation throughout imbibition

RNR expression was analysed by RNR:GUS localisation in high quality seeds (top row) and in seeds following accelerated ageing (bottom row) at the following stages: dry seeds, 6h imbibed seeds, seeds following coat cracking and after germination. A minimum of 15 seeds were isolated per condition.

RNR:GUS expression did not exhibit the same patterns of localisation or temporal expression as observed for *PARP2* (Figure 5.6). Whilst high quality seeds showed no evidence of RNR:GUS activity in the dry seed, staining was visible in the cotyledons of the aged seeds. The staining in cotyledons intensified in aged seeds following imbibition for 6 hours whereas only low levels were visible in high quality seed at 6 hours. High quality seeds also showed expression in the cotyledon following seed coat rupture where the lower quality seeds displayed markedly lower levels compared to earlier in imbibition. Furthermore the aged seeds showed intense staining towards the radicle, however the expression was concentrated between the shoot apical meristem (SAM) and root meristem rather than within the RAM. Localisation and abundance of *RNR:GUS* transcription was similar between the high and low quality seeds at germination (Figure 5.6d) with relatively low levels throughout the embryo, however previous transcriptional results would suggest there should be markedly lower presence following ageing. Aged

seeds displayed more pronounced staining of the root tip and SAM, sites where cell division is likely to be initiating and reaching high levels.

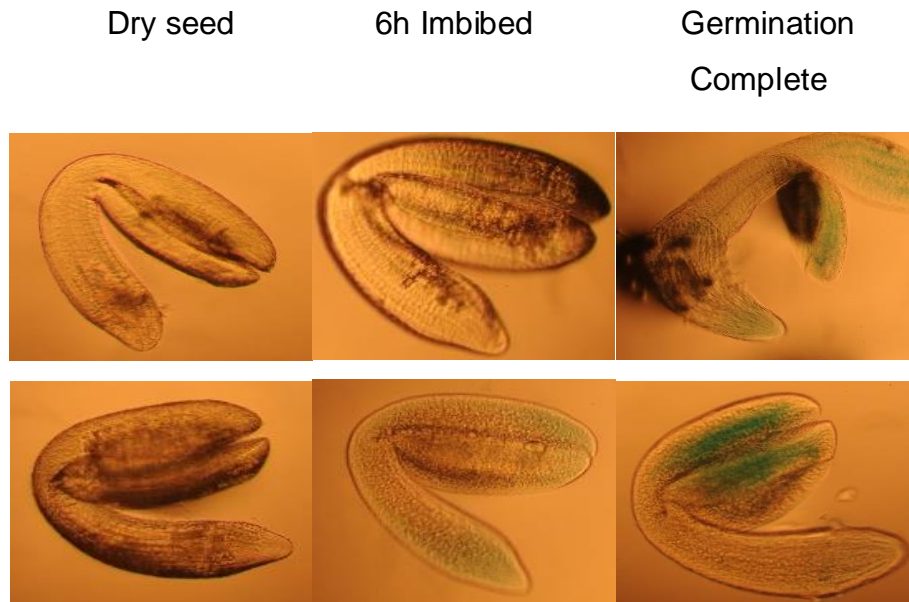


Figure 5.8 TK:GUS localisation throughout imbibition

The pattern of *TK* promoter activity was analysed by TK:GUS localisation in high quality seeds (top row) and in seeds following accelerated ageing (bottom row) at the following stages: dry seeds, 6h imbibed seeds, seeds following coat cracking and after germination. A minimum of 15 seeds were isolated per condition.

Thymidine Kinase is involved in the generation precursors for repair and replicative DNA synthesis. TK:GUS expression was undetectable in dry seeds and only became apparent during early imbibition, with low levels visible in cotyledons of high quality seeds and aged seeds, with evidence of staining in the root tip of the aged seeds. Following germination TK:GUS expression levels were much higher in cotyledons in aged seeds and less apparent in the root tip of aged seeds. During germination TK:GUS expression was visible in the root tips of high quality seeds, where aged seeds had shown levels after only 6 hours, and remained visible in the cotyledons.

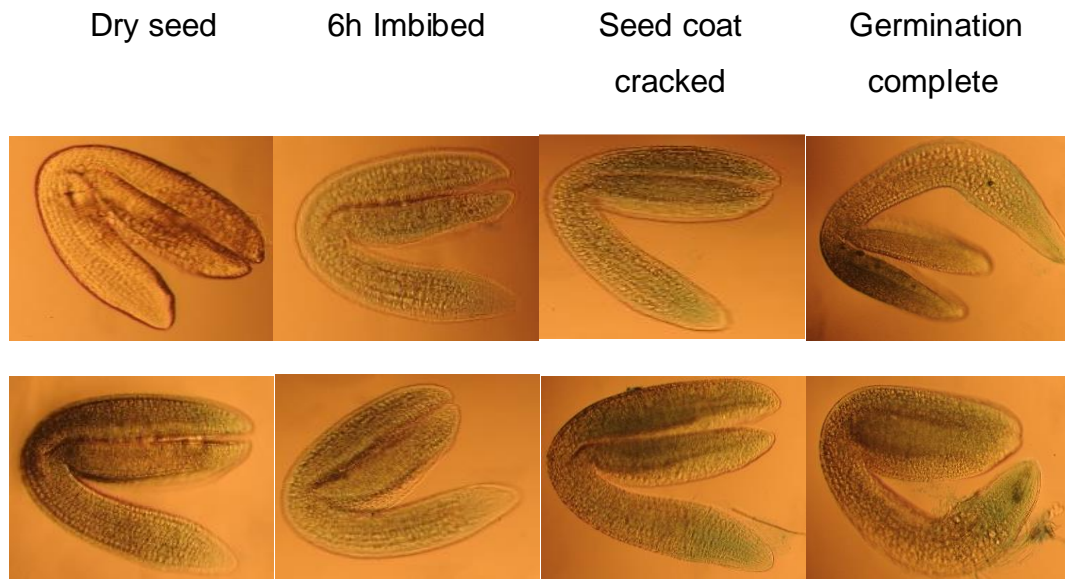


Figure 5.9 WEE1:GUS localisation throughout imbibition

WEE promoter activity was assayed using WEE:GUS localisation in high quality seeds (top row) and in seeds following accelerated ageing (bottom row) at the following stages: dry seeds, 6h imbibed seeds, seeds following coat cracking and after germination. A minimum of 15 seeds were isolated per condition.

WEE1, unlike TK, RNR and PARP, is responsible for delaying the cell cycle from entering S-phase, particularly under conditions of replication stress (ref). WEE1:GUS enzyme activity was not detectable in dry, unaged seeds but low levels are observed throughout dry seed following accelerated ageing (Figure 5.1A). Early in imbibition levels in the aged seeds do not change, with low levels visible throughout the seed. However this is where accumulation begins in the high quality seeds with low levels throughout the embryo which can be seen persisting through to the point of seed coat rupture. At this stage, GUS activity in high quality seed expressing WEE1:GUS shows a slight enhancement of staining in the root tip, a change that is more apparent following ageing with WEE1:GUS levels confined more towards the root meristem. Levels are similar through to germination with cotyledons and cells around the root tip showing low levels of WEE1:GUS expression in both high and low quality seeds.

5.3 Discussion

5.3.1 Abiotic stress and DNA damage in seeds

Orthodox seeds undergo a period of desiccation following maturation, with moisture content typically dropping to 10-15% without further damage (Ellis and Roberts, 1980; Franchi et al., 2011). The low moisture content allows for a significant reduction in metabolism to very low levels to help reduce energy requirements during prolonged storage. The low water content in seeds is associated with macromolecules in a glassy state that do not form ice crystals, thereby providing resistance to desiccation and freezing (Koster, 1991). During this dry period any damage arising from reactive oxygen species start to accumulate in cell compartments due to the low levels of repair activities. Respiration starts fairly rapidly upon rehydration in the seed, releasing the ROS from cellular compartments following the influx of water causing large amounts of oxidative stress to the seed (Ehrenshaft and Brambl, 1990; Tripathy and Oelmüller, 2012). This stress is linked to severe damage to macromolecules, including DNA, which has to be repaired sufficiently before the seed can initiate cell cycle and germination can begin (Elder and Osborne, 1993; Waterworth et al., 2016). The large amounts of potential damage during early imbibition are countered by the initiation of different repair processes. Some of these processes are designed to limit damage, such as enzymatic antioxidants which 'mop up' free radicals whereas some are responsible for repairing the inevitable damage that occurs, such as the DNA damage response (DDR).

5.3.2 The plant DNA damage response (DDR)

The DDR is a rapid response to DNA strand breaks activated in early imbibition (Culligan et al., 2006; Waterworth et al., 2010). The process is regulated by two phosphoinositide-3-kinase-related protein kinases (PIKKs): ataxia telangiectasia mutated (ATM) is the main component with minor roles suggested for ataxia telangiectasia mutated and Rad3-related (ATR). The activation of the DDR through these kinases has multiple downstream effects including; cell cycle arrest, histone modification, programmed cell death (PCD) and the transcriptional induction of hundreds of DNA repair genes. These

responses can be activated dependent on the severity of degradation and the specific tissue type, with programmed cell death (PCD) initiated in severely damaged stem cells in order to prevent mutation incorporation into the germline (Fulcher and Sablowski, 2009). In the case of double strand breaks uncertainties arise around the timing of the initial response between ATM and the MRN (Mre11, Rad50 and Nbs1) complex. MRN is involved in DSB end processing early in DSB formation and is necessary for ATM activation, however roles both upstream and downstream of ATM activation have been shown in MRN (Uziel et al., 2003; Lee and Paull, 2005; Amiard et al., 2010).

The transcriptional induction of DNA repair-related genes following imbibition is a specific response to double strand breaks, rather than a generic response to DNA damage, activated by the ATM-dependent DDR pathway (Molinier et al., 2005; Waterworth et al., 2010). Following γ -ray doses of 100Gy, to induce double strand breaks, DNA repair gene transcripts can be seen to increase as much as 500-fold, with hundreds of genes showing upregulation (Culligan et al., 2006). Several genes also are seen to be down-regulated during this response, notably those involved in G2 or M phase of the cell cycle, indicative of cell cycle arrest which has been shown to be a response to high levels of DNA damage (Ricaud et al., 2007). Altering levels of gene transcripts in times of stress is a highly conserved process that occurs across a wide variety of organisms; however the specific genes that are involved are poorly conserved across kingdoms (Culligan et al., 2006). For example the DNA repair-related gene BRCA1 is found in bacteria, plants and mammals but the transcriptional induction of this gene following DNA damage is only found in plants and bacteria and little change is found in humans (Rieger and Chu., 2004; Culligan et al., 2006).

5.3.3 Transcriptional induction of DNA Repair related genes in *Arabidopsis* during imbibition

The influx of water and resumption of respiration is an intensely stressful period of time for the seed. DNA damage and double strand breaks are detected in seeds during early imbibition, and levels increase after seed storage in unfavourable condition such as high heat and humidity, associated with loss of viability (Cheah and Osborne, 1978). The DDR is active as early

as three hours into imbibition, as large increases in transcripts coding DNA repair proteins are seen (Waterworth et al., 2010). Amongst these transcripts are *PARP2*, *RAD51*, *RNR* and *TK*, which are all involved in DNA repair processes and important for rapid germination. The levels of these transcripts rise in early imbibition before decreasing steadily towards the point of germination (Waterworth et al., 2010). This response reflects the accumulation of DNA damage during storage and activation of repair pathways early in imbibition. The level of transcriptional induction varies between genes. Here, the expression of well characterised DDR genes (*PARP2*, *RAD51*, *RNR* and *TK*) was characterised in seeds of different quality to identify any correlation between amount of damage and expression of repair genes.

Dry seeds had low levels of DNA repair gene transcripts in all conditions, in line with transcriptomic analysis (Waterworth et al., 2010). No significant effect of maternal environment on the DDR expression patterns was observed in any of the seeds tested; seeds produced from low, control and high maternal temperatures all displayed similar levels of transcripts across imbibition, and the same levels in high and low (aged) quality seeds. The different DDR genes varied in expression. The time of maximal transcriptional induction was later in imbibition for *RNR* and *TK* transcript levels whereas *PARP* displayed elevated transcript levels during both early and late imbibition and *RAD51* showing large increases during early imbibition before reducing again as the seeds reach germination. The late induction of *PARP*, *RNR* and *TK* suggest these factors are not required immediately upon imbibition, but act at a later time point and may be associated with the onset of S-phase. The seed lots germinated from around 67 hours after imbibition in the case of unaged seeds grown in high temperature (HT) conditions (section 3.2.2). The early imbibition time-point represents a time where germination is only 9.0% complete, although water uptake and seed water content should have reached a steady level (EcoSeed partner Finch-Savage lab, University of Warwick). However the likelihood is that transcript levels that are not present at 6 hours may be increased between the EI stage and the late imbibition (LI) time-point.

RAD51 was the only gene that showed high induction at the early imbibition time point. *RAD51* is involved in homologous recombination (HR); one of the

two major double stranded break repair pathways. Levels of RAD51 in seedling root tips increased over 100-fold in *Arabidopsis* following 100Gy γ -ray irradiation (Culligan et al., 2006). The localisation in root tips is consistent with HR utilisation being restricted mostly to dividing cells, given the requirement for HR in S-phase. High RAD51 levels very early in imbibition are seen consistently in high quality seeds (Figure 5.1); however the role of HR in seeds is unclear due to the cell cycle not being initiated until days later; around the time of germination. DSBs are typically resolved by non-homologous end joining (NHEJ) in non-dividing plant cells, however NHEJ transcript levels are not responsive to genotoxic stresses, likely due to constitutive expression of the NHEJ pathway.

5.3.4 Transcriptional induction of the DDR in aged seeds

High quality seeds are capable of initiating the transcriptional induction to generate large numbers of proteins associated with repairing damage that occurs during storage. However, the effects of seeds storage for long periods of time or in unfavourable conditions on the transcriptional DDR were unknown. Transcripts levels of three DDR genes: *RAD51*, *RNR* and *TK* were therefore compared between aged and unaged seeds during imbibition.

Poor quality seeds have an increased amount of DNA damage present which must be repaired sufficiently before germination proceeds (Dourado and Roberts, 1984). Despite the increased need for repair in aged seeds, the DDR transcriptional response was reduced rather than increased when compared to high quality seeds (Figure 5.4). Oxidation-mediated protein degradation in *Arabidopsis* does not instigate a rise in transcripts to replace the damaged proteins, instead preference is given to accumulating anti-oxidant defence mechanisms (Baxter et al., 2007). The lack of DDR mediated transcriptional response in aged seeds may therefore represent a preferential antioxidant response in a response similar to that of protein degradation, however the true reason behind the drop in transcripts is unclear.

The reduced transcriptional response in aged seeds may reflect the reduced capacity for *de novo* mRNA synthesis resulting from lower energy levels and loss of sub-cellular compartmentation (Kranner et al., 2010). Reduction in

germination vigour is associated with decreased protein synthesis capacity, which may reflect the energy status or transcript availability (Galland and Rajjou, 2015). The levels of mRNA do not correlate with levels of protein synthesis in seeds (Galland et al., 2012). The accumulation of damaged mRNA may also underpin the reduced levels of protein synthesis and aged seeds, and turnover of damage transcripts provides a possible explanation for the attenuated transcriptional DDR observed in aged seed lots. Alternatively, damaged transcripts may be difficult to detect using RT-PCR approaches if cDNA synthesis is inhibited. Northern analysis or array-based approaches that rely on hybridisation for transcript quantification (rather than reverse transcription) would resolve this technical difficulty.

5.3.5 GUS reporter localisation of DDR gene expression

Stable transformants with the β -glucuronidase (GUS) reporter gene under the control of the promoters of selected genes involved in the DNA damage response were generated. This allowed the expression pattern of repair genes to be localised, revealing cell- and tissue specific expression. The expression of four genes were analysed using GUS as a reporter. The first three; PARP, RNR and TK are involved in the DNA damage response and are required for repair. The fourth gene, WEE1, is regulated by both ATM and ATR as a result of DNA damage and replication stress and is responsible for halting cell cycle progression (Eckardt, 2007b).

There were two main areas where GUS expression is highest across the four genes; the cotyledons and the root tips. The root tips are the region where the cell cycle is initiated first, around the time of germination, and ensuring high levels of DNA integrity is important to avoid mutations contributing to subsequent cells and promote meristem activity. Cotyledons fall into two categories dependant on the type of plant. Some plants, such as legumes, have cotyledons with extensive nutrient storage to promote growth following germination. Other plants, including *Arabidopsis*, have cotyledons that elongate immediately upon imbibition and display cell division activity after a few days (Stoyanova-Bakalova et al., 2004).

Three of the four genes showed no GUS expression in high quality, dry seeds with only PARP showing detectable GUS levels in the cotyledons prior to imbibition. The low metabolic activity of dry seeds prevents any major changes in protein levels (Galland and Rajjou, 2015). Following ageing however, RNR and WEE1 GUS activity increased in dry seed, ruling out maternal synthesis of the DNA repair genes for dry storage in these instances. The changes in GUS levels in dry seed are unusual because of the lack of metabolism. However the proteins have been shown to increase and decrease in abundance after controlled deterioration, although the mechanism is unknown (Rajjou et al., 2008). For example, several proteins involved in the glycolytic pathway increase in dry seeds following ageing, a pathway that has been shown to be inducible through oxidative stress (Rajjou et al., 2008).

WEE:GUS expression was ubiquitous throughout the seed with higher levels found towards the root tip in aged seeds where the seed coat had cracked, that were not visible in high quality seeds, suggesting a delay to cell cycle initiation following ageing. Accelerated ageing is known to cause DNA damage and WEE1 presence in the root tip would indicate replication blocks in place early in imbibition. Increased levels in the root tip suggest a delay in the cell cycle to repair large amounts of damage before root tip proliferation despite levels of WEE1 transcript overall in seeds showing no significant difference in abundance (Waterworth et al., 2016).

RNR:GUS showed expression in the cotyledons in dry, aged seeds which increased during early imbibition. Expression was higher in the roots during seed coat cracking, especially in aged seeds, although the localisation was not focussed around the root tip which would be where most DNA repair synthesis would be expected to occur. Instead RNR:GUS was concentrated to the region between root meristem and shoot meristem before gradually focussing towards the two meristems following germination. Levels of RNR:GUS activity in the cotyledons were maintained throughout imbibition and in dry seeds that had undergone ageing suggesting RNR is important during all stages of imbibition and during maintenance in aged seeds (Figure 5.6).

Overall the levels of GUS expression, and thus promoter activity, did not correlate with RT-PCR analysis of *PARP*, *RNR* and *TK* transcript levels. Considering transcription is not required for completion of germination this may cause differences between the two methods, where promoter activity within the seed does not account for already stored mRNA molecules. Aged seeds often showed an increase in staining intensity in GUS localisation studies but displayed a reduction in transcript levels in RT-PCR approaches. Few of the conditions showed an increase in GUS activity in high quality seeds, including thymidine kinase, which displayed high GUS activity in unaged seeds around the root tip following germination. This trend could be accounted for due to the need for new thymidine molecule generation for incorporation into the genome during replication which would be more efficient in seeds of high vigour with seedlings generally growing faster and thus increasing cell numbers.

6. Detecting oxidative DNA damage in seeds

6.1 Introduction

Base damage occurs on single nucleotide molecules and can cause mutagenesis without necessarily affecting any surrounding nucleotides. Oxidation is thought to be the primary type of damage occurring in the aged orthodox seed genome mainly due to desiccation and rehydration cycles generating large amounts of reactive oxygen species (Bailly, 2004; Kranner et al., 2010; Considine and Foyer, 2014). There are several single-base lesions associated with oxidative damage, however the oxidation of guanine to produce 8-oxoG is the most prevalent form of oxidative lesion affecting nucleotides (Kanvah et al., 2010). These lesions require excision, primarily through the base excision repair (BER) pathway.

There are several products which can potentially cause oxidative damage throughout the cell. Free oxygen radicals such as the hydroxyl radical ($\bullet\text{OH}$) and non-radicals such as hydrogen peroxide (H_2O_2) are the major cause of damage, with their damage potential based on differences in redox potentials (Kawanishi et al., 2001). DNA bases show differences in oxidation potential, with guanine having the lowest and is therefore the most likely to be oxidised especially in sequences containing a guanine repeat (GG) (Boone and Schuster, 2002). The oxidised guanine molecules produced by reactive oxygen species show propensity to generate G:C to T:A transversions through misreading of replication machinery (Bjelland and Seeberg, 2003). Mutations in somatic cells of the root or shoot meristem prior to cell cycle initiation risk incorporating these mutations in cells throughout the plant.

The process of removing oxidised guanine lesions through BER generates apurinic/apyrimidinic (AP) sites which cause breaks in the phosphate backbone of DNA. AP sites in aged *Zea mays* seeds accounted for 38 in every 10^6 nucleotides and these single strand breaks can cause loss of genetic information and mutagenesis (Dandoy et al., 1987). 8oxoG is excised from the genome by one of two interchangeable DNA glycosylase enzymes: formamidopyrimidine [fapy]-DNA glycosylase (FPG) or 8-oxoguanine glycosylase (OGG) depending on species. All higher plants have both FPG and OGG with redundant roles in the excision pathway (Murphy and George, 2005), as evidenced by the increase in oxidative damage found in nuclear and

mitochondrial seedling DNA in the absence of these two enzymes (Córdoba-Cañero et al., 2014).

Oxidative damage to DNA can cause mutations directly without the AP intermediate. Oxidised guanine molecules can be misread by replication machinery as thymine and cause nucleotide transversions from G to T and subsequently C to A, resulting in mutagenesis (Figure 1.1). Repair machinery attempts to remove the affected nucleotide before cell cycle progresses. If cell cycle begins prior to removal of the afflicted nucleotide the mutation is replicated and permanently fixed within the genome.

6.1.1 Aims

To determine the prevalence of oxidation in the Arabidopsis seed genome novel methods were developed in order to measure the levels of oxidised guanine molecules. Measuring genome oxidation levels in seeds of different quality allows us to determine whether oxidation correlates with the reduction in germination capacity of selected seed lots. Comparing oxidation levels with seed performance in germination tests will determine whether the methods used to measure 8-oxoG offer sufficient sensitivity to be utilised as seed quality biomarkers.

6.2 Results

6.2.1 Measuring 8-oxoG as a quantitative biomarker for oxidative Stress

To determine the correlation of oxidative DNA damage with seed ageing, 8-oxoG levels were measured using PCR-glycosylase quantification of FPG cleavage activity at specific genomic loci. Oxidised guanine molecules are difficult to detect, however by removing these lesions using selected enzymes, in this case FPG, the resulting single strand breaks can be measured by the inability of polymerases to amplify the broken strands i.e. FPG mediated reduction in copy number. The amount of oxidation can then be quantified and correlated with seed quality. This will help to determine whether oxidation of the genome is a key marker of a seed lot to germinate.

A method was developed to measure the amount of 8-oxoG in different plant species at different imbibition time points to establish whether levels correlated

with seed performance. Genomic DNA was treated with the enzyme FPG to remove oxidised guanine molecules before PCR amplification. FPG creates a single strand DNA break through base excision and the associated lyase activity that cuts the phosphodiester backbone immediately halts amplification. The difference in amplification obtained between DNA that had been treated with FPG and control DNA incubated only in water allowed the quantification of 8-oxoG levels in aged seeds.

Initially the effectiveness and sensitivity of this PCR-glycosylase approach to determine levels of 8-oxoG in DNA was evaluated. To determine whether the PCR-glycosylase approach provided a quantitative measure of 8-oxoG levels, PCR products incorporating known amounts of 8-oxoG were synthesised and tested. PCR was used to amplify a DNA fragment using primers with and without an 8-oxoG present. Mixing and titration of PCR products allowed the production of DNA with a known amount of base modification (Table 2.1). Treatment of the product with the enzyme FPG results in cleavage of the DNA backbone and prevents amplification. PCR amplification of oligonucleotides that contained 8-oxoG yielded no final product following FPG incubation, suggesting that an abasic site is created in all strands containing the lesion, terminating elongation. Amplification of the oligonucleotide in the absence of FPG did however generate several copies, showing that DNA polymerase can efficiently read guanine as a nucleotide, even in an oxidised state. Furthermore, when combining ratios of oligonucleotides with known percentages of 8-oxoG there is high correlation between number of 8-oxoG molecules and final copy number (Figure 6.1) ($R^2 = 0.983$).

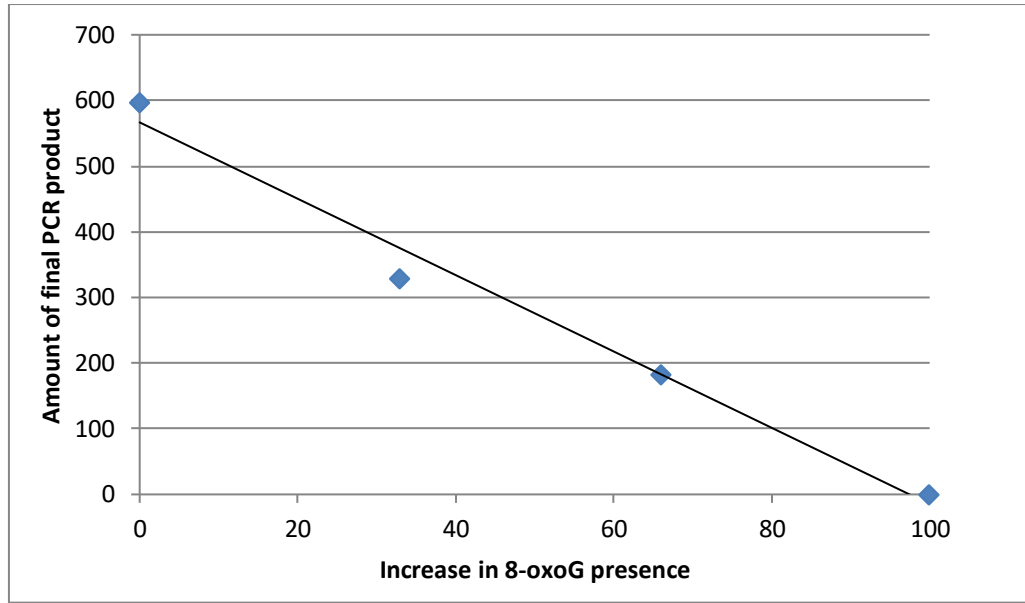


Figure 6.1 Correlation between 8-oxoG and final copy number following PCR amplification

To determine the efficiency of 8-oxoG cleavage by FPG in solution two sets of primers were synthesised; one containing an 8-oxoG and an identical set of primers with a regular guanine molecule (table 2.1). There was no amplified product in the final solution post-PCR when 8-oxoG containing oligonucleotides and FPG were combined, as measured by qPCR. The two oligonucleotides were combined in different ratios showing the increase in 8-oxoG presence correlated highly 8-oxoG levels ($R^2 = 0.983$), thus proving accurate quantification.

6.2.2 Designing primers for use in *Arabidopsis*

Orthodox seeds accumulate extremely high levels of DNA damage, much higher than at other stages of the plant lifecycle (Waterworth et al., 2016). For the technique to work effectively in seeds, primers would need to sensitively quantify guanine oxidation changes in the seed genome. Establishing the prevalence of genomic oxidation in seeds throughout imbibition would increase our knowledge on which stages of imbibition are most stressful and when most repair of oxidative DNA damage occurs. Primers were generated to amplify regions of the *ACTIN1* (*ACT1*) gene. Different length products were obtained and treated with FPG, larger PCR products contained more guanine molecules, and were therefore more likely to contain oxidised bases that would

cause termination of amplification. Studies with *Arabidopsis* seedlings suggests that the occurrence of 8oxoG is approximately two in every kilobase; however levels in seeds are expected to be much higher than in seedlings (Córdoba-Cañero et al., 2014). In line with this, amplification of DNA generating fragments higher than 1kb showed no amplification following FPG treatment, suggesting that at least one oxidised guanine molecule occurs in every 1kb of DNA (Figure 6.2). Sensitivity was very high with final primer selection amplifying a 198bp region for FPG treatment, as shown in Figure 6.3.

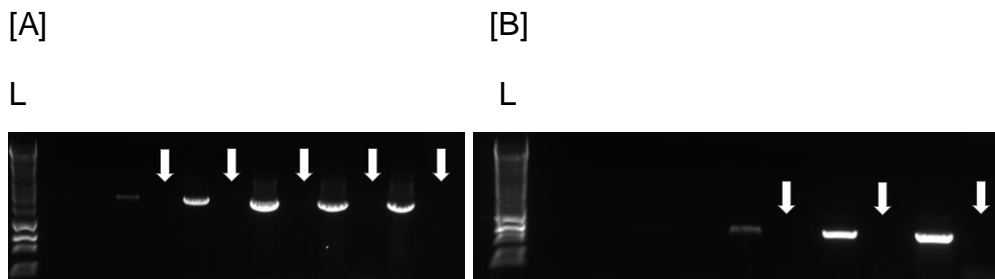


Figure 6.2 Amplification and FPG treatment to determine fragment size

DNA was extracted from *Arabidopsis* seeds and either remained untreated or was treated with FPG. Visible bands represent the untreated samples where amplification occurred and white arrows denote the lack of product following FPG treatment. Different primers were used to determine sensitivity of the method, with fragments >1kb in size failing to amplify following FPG treatment meaning at least one 8-oxoG is present per kilobase. The amplification products shown were 2kb [A] and 1kb [B] in length. Final fragment length selection was 198bp in size as it showed reduced amplification across a range of conditions (figure 6.3). Each band represents an increase in PCR cycle number ranging from 20 cycles to 40 cycles in incrementing groups of 5 cycles. L; Bioline Hyperladder 1kb.

6.2.3 Effect of DNA quality and quantity on the frequency of 8-oxoG Excision

Extraction of high quality DNA from seeds can often prove difficult because of the large quantities of polysaccharides and storage compounds present (Xin and Chen, 2012). Optimisation of DNA extraction from seeds for analysis of DNA damage is described in Chapter 4. These by-products of extraction are often co-precipitated with DNA and cause a reduction in yield and contamination of DNA. These impurities often lead to difficulties determining the accurate concentration of DNA when using spectroscopy and interfere with DNA migration in electrophoresis (Chapter 4). The presence of these impurities might therefore also interfere with FPG-PCR mediated quantification of base damage. This was assessed by determining whether Arabidopsis DNA extracts displayed concentration dependence when determining levels of 8-oxoG. DNA was extracted from 48-hour imbibed Col-0 seeds and was left either undiluted or was diluted 4 times or 8 times with ddH₂O. The samples were digested with FPG as described in section 2.8.2 and quantification carried out as outlined in section 2.9.1.

There was little difference in final copy number reduction following dilution and FPG treatment. The DNA, when left undiluted, had a 39.4% reduction in copy number which changed narrowly to 42% reduction when diluted 8x. The dilutions used were prepared to provide accurate readings as possible when using agarose gel quantification. Further dilutions gave no product after FPG treatment and lower copy numbers generate weaker fluorescent bands which are more difficult to quantify accurately.

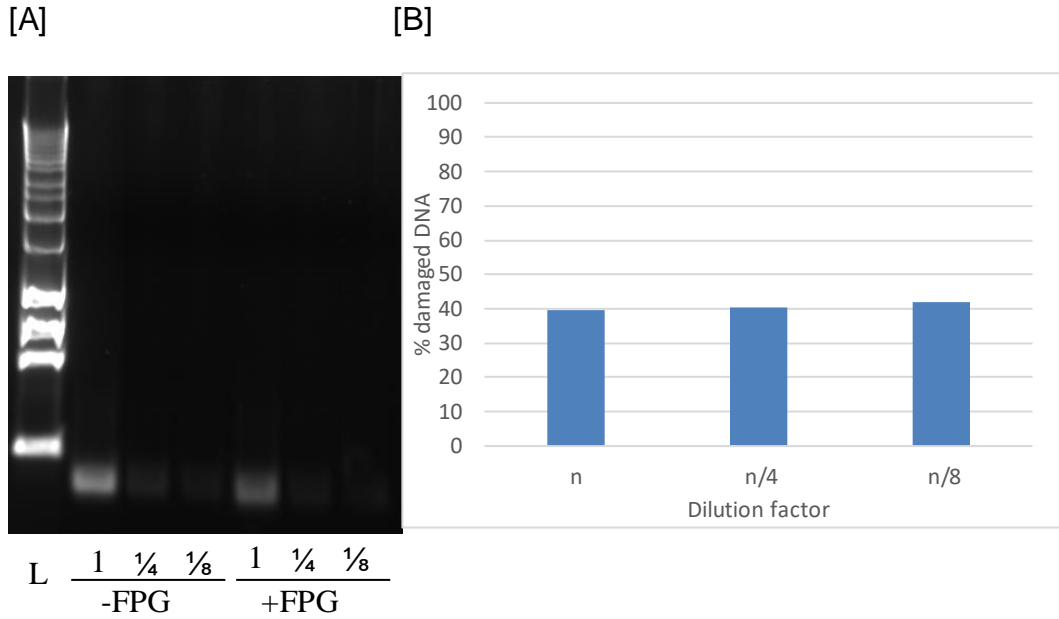


Figure 6.3 Reduction in copy number of FPG-treated DNA following dilution and PCR amplification

DNA was extracted from 48 hour imbibed Col-0 seeds and was left undiluted or diluted 4-fold and 8-fold with ddH₂O following FPG treatment. The products were run on an agarose gel and reduction in copy number quantified by fluorescence of bands.

[A] Agarose gel showing PCR product of undiluted DNA (1), DNA diluted 1 in 4 with ddH₂O (1/4) and DNA diluted 1 in 8 with ddH₂O (1/8) with (+FPG) or without (-FPG) FPG treatment. The ladder used was Hyperladder 1 kb plus (lane L).

[B] Graph showing the reduction in fluorescence of FPG treated samples when compared to those left untreated. There does not appear to be a correlation between dilution and final copy number with the undiluted sample showing a 39.4% reduction, the 4x dilution showing 40.4% reduction and the 8x dilution showing 42.0% reduction.

6.2.4 Repair of 8oxoG during imbibition of high quality *Arabidopsis* seed

In initial studies, temporal changes in levels of oxidised base damage throughout germination of high quality *Arabidopsis* seeds were evaluated. The causes of DNA damage in germination are incompletely understood and the re-entry of water upon rehydration may increase levels of oxidative DNA damage. DNA repair processes are believed to initiate early in imbibition (Elder and Osborne, 1993; Waterworth et al., 2010). Oxidation levels fluctuate throughout germination with little available knowledge on oxidation throughout imbibition and the causes of increases and decreases. Levels of 8oxoG were monitored throughout germination to establish at which point levels of genome oxidation were significantly low, signifying repair and reduction in DNA damage. Seeds were generated in greenhouses at controlled temperatures (22°C; 16 h:8 h day/night cycle) and kept as unaged or subjected to accelerated ageing for 1 day (35°C, 80% RH). The DNA was extracted and subjected to the same PCR-glycosylase treatment as previous (section 2.8 and 2.7.3). Aged seeds showed an increase in DNA oxidation compared to dry seed, with unaged samples displaying 52.1% oxidation increasing to 94.5% following 1-day ageing ($P < 0.05$). Following 9 hours of imbibition the levels of oxidation were at their lowest in both the unaged (10.7%) and in aged samples (38.6%) ($P < 0.05$). Although oxidation levels in the aged seeds were at their lowest at 9 hours imbibition they were still significantly higher than in the unaged seeds ($P < 0.01$) (Figure 6.4).

It is important to increase knowledge of the molecular basis behind differences in seed quality, in order to effectively generate biomarkers for seed quality then such knowledge must be applied in an affordable and widely accessible way and be transferable across a number of species. As this is an ultimate aim of the EcoSeed project, we modified the methodology involved in the quantification step from the relatively expensive qPCR quantification to agarose gel quantification (Section 2.9.1). The reduction in transcript levels will be measured by the drop in fluorescence similar to the quantification method used in Figure 6.4. Comparable results were obtained using agarose gel quantification as with qPCR quantification, on average showing $\pm 6.8\%$

variability, increasing the accessibility of using quantification of oxidised as a biomarker.

We also developed a method of estimating base damage based on the percentage of damaged transcripts. As we know the size of the *Arabidopsis* genome, and assuming only one oxidised guanine molecule appears in each 198 bp long transcript that has failed to amplify, then it is possible to extrapolate the number of damaged bases across the whole genome. The equation allows for a new way of presenting data that increases accessibility to a wider audience (Figure 6.4).

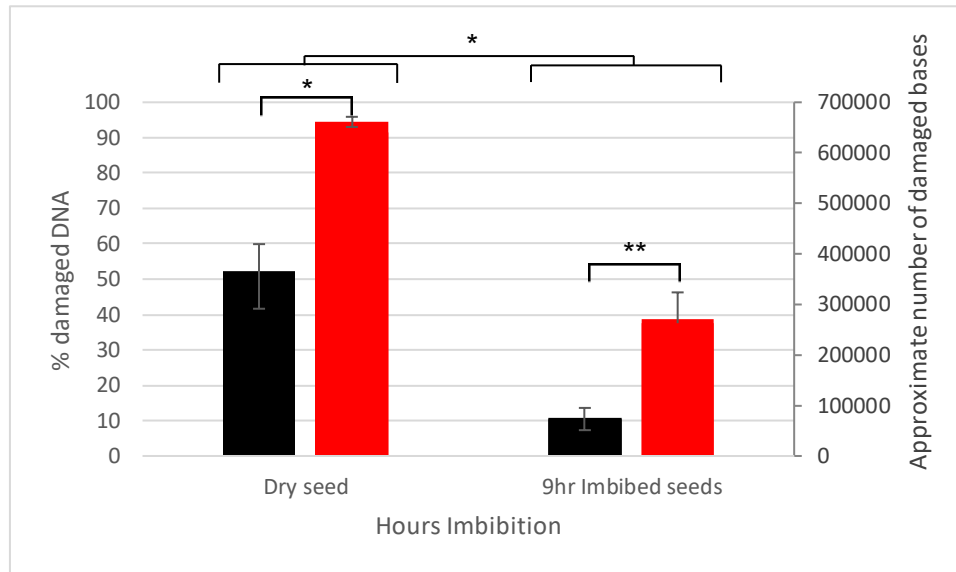


Figure 6.4 Base oxidation during ageing and reduction following imbibition

Aged seeds (■) that had not been imbibed showed an increase in 8oxoG content of 42.3% when compared to high quality seeds (■), suggesting an increase in oxidative pressures in these seeds. Following imbibition for 9 hours there was a reduction in oxidation in both high quality and low vigour seeds. High quality seeds showed a 41.3% reduction ($P < 0.05$) following incubation, whereas the aged seeds dropped by 55.8% ($P < 0.05$). Although 9 hours imbibition resulted in a drop in oxidation in both aged and unaged seeds, the aged samples still had significantly more guanine oxidation ($P < 0.01$).

By knowing the proportion of oxidised products in a 198bp region of *Arabidopsis* DNA, this number can be extrapolated to represent genomic oxidation allowing for a different representation of the work. This method gives approximate numbers of oxidised lesions at the genome level and is shown on the secondary y-axis.

6.2.5 Oxidation in non-transcribed regions of DNA

DNA oxidation occurs across the whole genome and large amounts can be found in seeds, as shown previously. The previous sections of this chapter used a small region of the Actin1 gene in order to estimate numbers of oxidised guanine molecules across the genome. However, transcribed regions only account for a small percentage of nuclear DNA and some repair processes preferentially repair actively transcribed regions, such as lesions linked to transcription (TC-NER). DNA damage products can stall the progression of the transcription complex on the DNA template, compromising gene expression and downstream protein synthesis. Damage and repair may therefore differ at these loci relative to the rest of the genome. ACTIN1 is a protein found widely across many plant species and is actively transcribed in seeds (Hightower and Meagher, 1986); it is unclear whether the transcriptional activity around this gene would influence levels of oxidative damage. DNA from three sources were therefore used in order to test the oxidation in a gene-coding region of *ACTIN1* and another in a non-coding area of chromosome 1. High quality dry *Arabidopsis* seeds, dry seeds that had been aged for one day and aged seeds that had been imbibed for 9 hours were amplified using the two primers to determine whether there were any oxidative differences in the two regions. The 9-hour imbibition time point represents a time when transcription of *ACTIN1* will have started and has been shown to be a period of time where repair of oxidation is high (section 6.2.5).

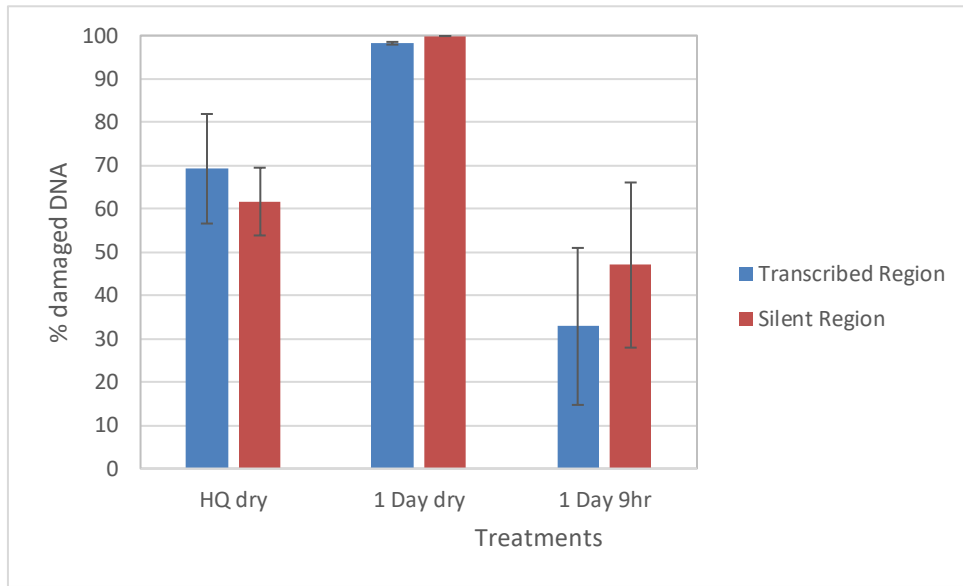


Figure 6.5 Genomic oxidation in silent and protein coding regions

Identical sized oligonucleotide fragments were generated using primer amplification of two regions; one involved in the transcription of the *ACTIN1* gene and one in a non-coding area of DNA. These fragments were subjected to the same FPG treatment as described (section 2.8.2). There was no difference between the two regions in terms of guanine oxidation in unaged seeds, aged seeds or following imbibition.

High quality dry seeds showed a 69% reduction in final copy number of the Actin region following amplification and the silent region showed a similar 62% reduction. Aged seeds showed no difference between the two regions with a 98% reduction in *ACTIN7* copy number and a 99% reduction in the non-coding region. Because of the reduction in oxidation seen following 9 hours imbibition, and the active transcription of the genome at this time, we used this time point to determine whether repair is lower in silent regions. There appeared to be no preferential oxidation removal in the active region compared to the silent region, with a reduced level of oxidation in both.

6.2.6 The effect of maternal environment and post-harvest ageing on 8oxoG accumulation in seeds

The aim of determining oxidative damage levels is to discover whether accumulation affects overall seed quality. DNA was extracted using a chloroform based extraction (section 2.6.2) from *Arabidopsis* seeds that had been produced in different maternal environments, low temperate (LT; 14°C/16°C), control temperature (CT; 18°C/22°C) and high temperature (HT; 25°C/28°C) (full details on conditions in section 3.2.2). The seeds were also subjected to accelerated ageing to determine how storage affects the accumulation of oxidised guanine (40°C, 75% RH for 4 days). The DNA was then subjected to an FPG enzymatic reaction and cycled through a qPCR machine for quantification as described in section 2.7.3.

In dry seeds there was no difference in oxidised guanine content between seeds grown under any of the three maternal conditions. Seeds grown under HT did not differ following accelerated ageing, with 35.7% damaged transcripts in unaged seeds and 35.0% following ageing. Control temperature seeds also showed no significant difference following ageing ($P=0.11$). Seeds grown in a lower temperature showed more oxidation following ageing, with unaged seeds having 54.6% oxidation levels compared to the higher 88.7% levels following ageing ($P<0.05$) (Figure 6.4).

Early imbibition (6 hours) represents a time where water uptake is steady and respiration enables initiation of repair processes. LT seeds showed repair of oxidised guanine molecules by 6h, with levels dropping by 32.1% following imbibition ($P<0.05$). LT seeds showed the lowest levels of base oxidation in early imbibition (22.5%); lower than both CT seeds (69.7%) and HT seeds (48.1%). Aged seeds from the control group (CTA) increased in oxidation following imbibition by 40.9% ($P<0.05$). There was no significant difference in seed lots that were aged compared to their respective unaged seeds.

Late imbibition (72 hours) represents 80% of the mean germination time for CT seeds and can be used to look at how damage levels change as the seed prepares to initiate cell cycle and start germination. Oxidation levels in LTA seeds remained reduced from the dry seed (unchanged from early imbibition)

whereas CTA seeds showed a 67.4% increase in 8-oxoG levels compared to the dry seed. HT seed DNA oxidation increased from early imbibition by 13.0%. There was once again no difference in oxidation between ageing treatments in any of the seed samples.

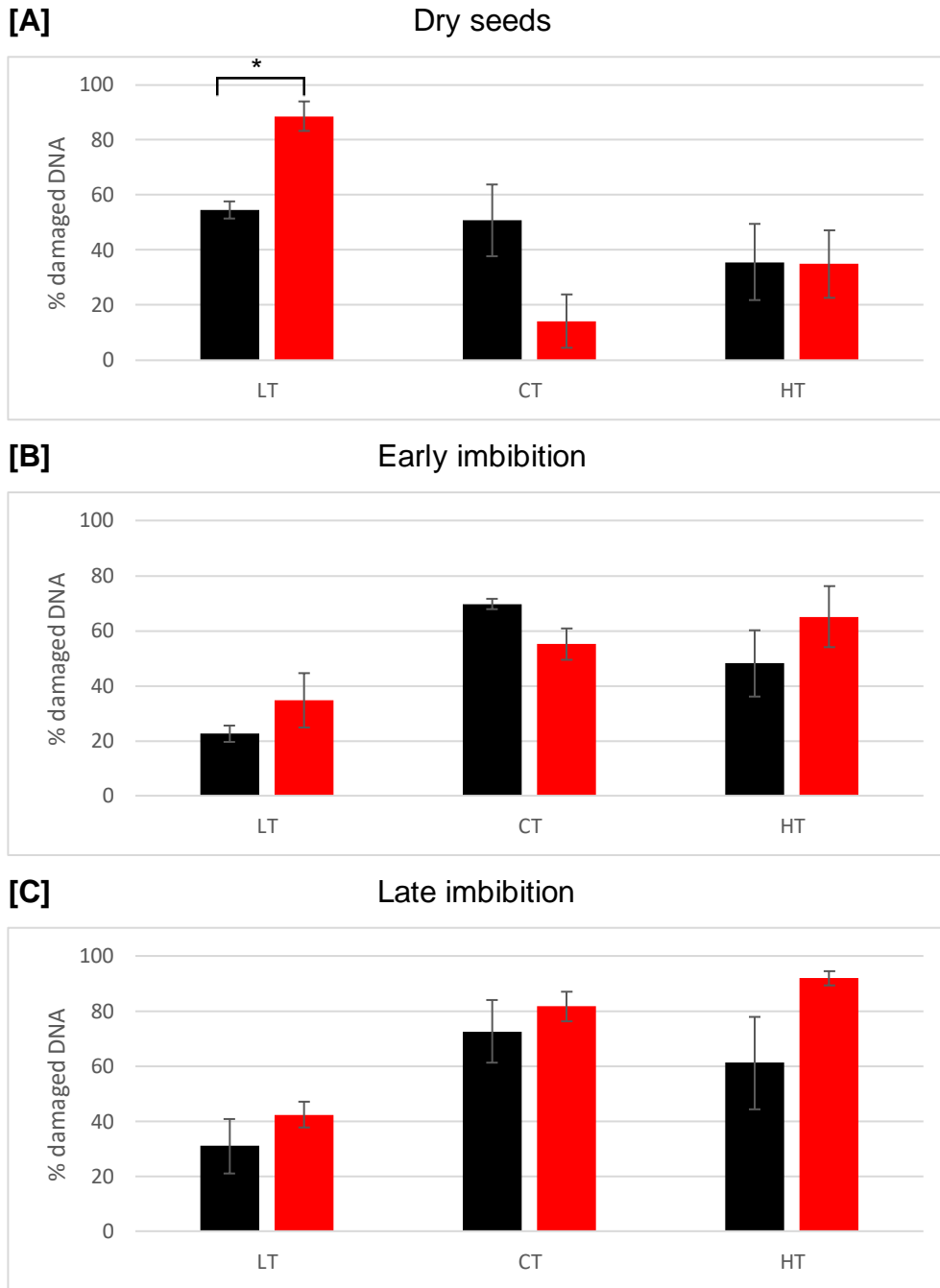


Figure 6.6 Effects of the maternal environment and accelerated ageing on 8-oxoG accumulation in Arabidopsis seed imbibition

8-oxoG accumulation across different imbibition time points was measured in *Arabidopsis thaliana* seeds of high quality (■) and seeds subjected to controlled deterioration (■)

[A] 8-oxoG levels in dry seed There were no significant differences in base oxidation in unaged seeds produced at different maternal temperatures in (LT, CT, HT). However LTA seeds showed much greater levels of oxidation (88.7%) than CTA

(14.2%) or HTA (35.0%) seeds ($P < 0.05$). LT was also the only seeds to show significant difference following ageing, increasing by 34.1% in LTA seeds ($P < 0.05$) compared to the respective unaged treatment.

[B] 8-oxoG levels during early seed imbibition LT seeds significantly repaired base damage following imbibition for 6 hours, showing decreased levels in comparison to dry seed ($P < 0.05$). LT seeds also maintained lower levels of 8-oxoG than CT seeds, showing 47.2% lower levels ($P < 0.01$). There was no significant change in 8oxoG presence in any of the seed batches following accelerated ageing.

[C] 8-oxoG levels during late imbibition LT seeds remained at the lower levels as seen in early imbibition, however levels were not significantly different than CT seeds following imbibition for 72 hours. CTA seeds had a larger accumulation of 8-oxoG during late imbibition in comparison to dry seed. Accelerated ageing had no effect on 8-oxoG levels.

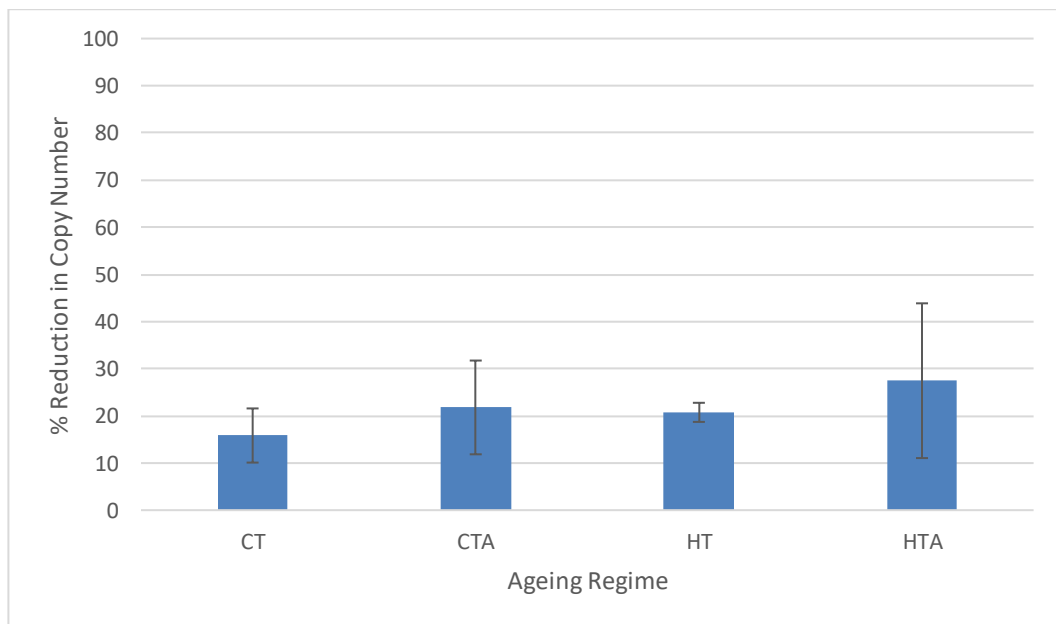


Figure 6.7 Reduction in copy number following FPG incubation in dry *Brassica* seeds

Quantification of oxidative DNA damage in *Brassica* seeds. Neither maternal environment nor ageing caused significant change in oxidised base levels in dry *Brassica* seeds. Levels were much lower than those seen in *Arabidopsis* suggesting more robust protective mechanisms may be present in *Brassica* seeds. Ageing regimes are as described in (2.5.4)

6.2.7 Effects of the maternal environment and accelerated ageing effects on 8-oxoG accumulation in *Brassica* seed

The levels of 8-oxoG were next determined in *Brassica* seeds produced under control (18°C/22°C; CT) and high (25°C/31°C; HT) maternal temperatures temperature regimes to investigate the effects of maternal environmental temperature on levels of oxidative base damage. *Brassica* seeds were also analysed for presence of oxidised guanine molecules either before (CT, HT) or after (CTA, HTA) ageing in the same way as described above (section 6.2.4). Seeds that were produced at the control temperature showed a 15.8% reduction in copy number, with no significant difference after ageing (21.7% reduction). Seeds produced at a higher temperature did not have significantly more oxidative damage, nor did the same seedlots show any difference after ageing, with a 20.7% and a 27.4% reduction in final copy number respectively. There was also no observable difference between seeds produced in different maternal environments.

6.3 Discussion

6.3.1 Determining the efficiency of FPG to remove 8-oxoG

From early work in mammalian and bacterial genomes the levels of genomic oxidation were always estimated to be several fold lower than that of mitochondrial DNA. More recently, partially due to updated methodology, the levels of genomic oxidation are now seen to be similar to that of mitochondrial DNA (de Souza-Pinto et al., 2001; Trapp et al., 2007).

The discrepancies arose through early studies utilising chromatographic methods to determine oxidation. Both mass spectrometry and high pressure liquid chromatography (HPLC) are vulnerable to artificial oxidation events during isolation of DNA and subsequent hydrolysis, leading to suspected over-estimates in DNA oxidation (Collins, 2005). Considering the significant contribution macromolecular oxidation has on the seed ageing process, we developed a non-chromatographic method in an attempt to limit levels of artificial oxidation sites introduced in analysis. The differences in oxidation levels between the two methods vary, chromatographic methods generally suggest between 5 and hundreds of oxidation residues per 10⁶ guanines,

whereas enzymatic methods (such as employed here) tend to suggest ~0.5 oxidation events per 10^6 guanines (Collins, 2005).

As a control to determine how quantitative the FPG-PCR assay is, 8-oxoG containing primers were generated to allow introduction of known levels of 8-oxoG into DNA. These primers allowed the introduction of 8-oxoG into oligonucleotides which, upon excision using a dual AP-lyase/glycosylase (FPG), would fail to amplify using PCR. Combining products known to contain the excised bases (incapable of amplification) and whole-fragments (those capable of amplification) allows us to generate a template DNA with a known percentage of 8-oxoG containing DNA strands. Two templates were made; one containing no excised fragments which should amplify fully and one containing only excised fragments which should be incapable of amplification. The two were also combined to generate solutions containing 33.3% and 66.7% excised fragments to determine the relationship between percentage base excision and final copy number. The result was a line of best fit with an R^2 value of 0.983, showing that the levels of PCR amplified product is inversely correlated to the presence of oxidised guanine molecules.

6.3.2 The percentage of 8-oxoG in DNA can be measured over a range of template concentrations

The methodology was then transferred to seeds to compare oxidation in seeds of different quality. Due to the co-purification of large amounts of carbohydrates during DNA extraction protocols, it is difficult to accurately determine DNA concentrations and it was unknown how 8-oxoG excision would be affected by the amount of DNA present. Three dilutions were made of extracted seed DNA that had been imbibed for 48 hours; seed DNA at ~750ng, the same DNA diluted four times in water and another diluted 8 times. There was no significant change between the three samples, with 1x DNA, the 4x dilution and the 8x dilution showing a reduction in copy number by 39.4%, 40.4% and 42.0% respectively. This indicates that the assay is robust and not likely to be influenced by small differences in starting quantity between samples.

6.3.3 Low temperature in maternal development increases oxidation of guanine upon ageing

Seed maturation conditions have been shown to affect how well seeds establish dependent on temperature (Koller, 1962), along with differences in seed size and viability (Mohamed et al., 1985). Higher temperatures during maturation are linked to a reduction in seed yield, with lower temperatures linked to an increase in dormancy (Section 3.2.2) (Kendall et al., 2011; Huang et al., 2014). By using seeds produced in different temperature regimes and subjecting them to accelerated ageing regimes, it is possible to see whether temperature during seed development can alter germination performance in combination with sensitivity to storage. There was no significant difference in the dry seed between the accumulation of 8oxoG in seeds produced in low (LT), control (CT) or high temperatures (HT). This indicated that, at least under the conditions and genotypes investigated in the present study, that maternal environment does not increase genomic oxidation prior to seed shedding (Figure 6.4). However, the maternal environment did influence how the seeds responded to ageing stress, as dry LT seeds demonstrated increased DNA oxidation after being subjected to accelerated ageing ($P < 0.05$). These seeds also showed poorest germination performance, with reduced vigour and viability (Figure 3.2) possibly as a result of increased oxidative pressure building up in dry seeds. This is exemplified by increasing levels of hydrogen peroxide (H_2O_2) in seeds grown in lower maternal temperatures, with ageing significantly increasing H_2O_2 presence (EcoSeed partner; Bailley lab, Pierre and Marie Curie University). Similarly, flavonoid concentrations increase at lower temperatures which are seen in plants undergoing high levels of stress (EcoSeed). The major increase is seen in quercetin flavonoid derivatives which are suggested to have a role in singlet oxygen scavenging (1O_2) (EcoSeed partner INRA, France)(Agati et al., 2012).

6.3.4 *Arabidopsis* seeds aged for one day show increased levels of oxidation

Quantification was carried out on *Arabidopsis* seeds that had been grown in greenhouses of controlled temperature (section 2.4) and had been aged at defined RH and temperature using a saturated salt solution of KCl (35°C; 80%

RH) as described in section 3.2.1. DNA was extracted from unaged seeds and seeds that had been aged for one day. Oxidative damage in dry seeds accumulates even with very low levels of metabolism and is exacerbated by the influx of water when imbibed as respiration initiates (El-Maarouf-Bouteau and Bailly, 2008). The acquisition of desiccation tolerance in orthodox seeds increases protection from oxidative damage and levels of base damage in dry seeds, stored for 2 years, is still 6 fold lower than DNA in aqueous solution (Dandoy et al., 1987). We suggest that oxidation is still high in the dry seed despite these protective mechanisms which is exacerbated during ageing (Figure 6.4).

The presence of water initiates the seed to begin metabolism and, although this is an additional source of stress for an orthodox seed, it allows for the activation of repair mechanisms. Genomic repair is essential in the early stages of imbibition due to the need for protein synthesis and the priority of reducing risk of mutation before the cell cycle starts. Should mutations persist when the cell cycle activates it is possible they may contribute to the germline of the whole plant. The presence of oxidation in the dry seed is increased with one day ageing when compared to high quality dry seeds. Using qPCR to measure copy number reduction (section 2.7.3) high quality seeds showed a 53.2% reduction in transcript levels, whereas one day aged seeds show a 96.7% reduction (Figure 6.4). This shows that under sub-optimal storage conditions oxidation is a major source of damage in dry seeds. The increase in oxidation as a consequence of controlled deterioration (CDT) has been shown previously, with imbibition also increasing the levels of 8oxoG (Chen et al., 2012). The high levels of oxidation were reduced by overexpression of OGG1, one of the two DNA glycosylases responsible for 8oxoG removal, which also saw a marked improvement in germination performance following CDT (Chen et al., 2012).

6.3.5 Lowest levels of 8-oxoG are found around 9-hours post imbibition

The early stages of imbibition are often seen as one of the more stressful events a seed encounters. Despite the increase in damage associated with water influx and initiation of metabolism, this period of time is also where repair of genomic damage occurs before cell cycle starts (El-Maarouf-Bouteau and

Bailly, 2008; Waterworth et al., 2010). Several responses early in imbibition are targeted towards ensuring the integrity of the plant's genome, such as transcriptional induction of DNA repair genes, delay to cell cycle induction and signalling of double strand breaks (Elder and Osborne, 1993; Waterworth et al., 2016). The levels of oxidative damage therefore flux throughout imbibition, increasing with water influx and respiration resumption but decreasing with repair activities (Waterworth et al., 2016).

During imbibition levels of oxidation reach their lowest levels after around 9 hours in both unaged seeds and 1 day aged seeds. This suggests that in the early hours of seed imbibition the repair of oxidised guanine molecules is less than the rate of formation of new lesions. Within the first 20 hours of imbibition (around 50% of the way to germination) the number of AP sites in aged *Zea mays* seeds increased 4-fold in comparison to dry seeds (Dandoy et al., 1987). The formation of these sites through glycosylase action demonstrates the large levels of genomic repair occurring throughout early seed imbibition to turn over damaged base formation.

At around 9 hours we discovered 8-oxoG levels to be at their lowest, indicating repair very early in imbibition. The synthesis of DNA-repair related transcripts within the first few hours of imbibition and the formation of additional AP sites suggest that this represents a time where genomic repair activities peak (Dandoy et al., 1987; Waterworth et al., 2010). The amount of oxidation in high quality seeds, as measured by drop in copy number, decreased by 2.5-fold from 53.2% to 21.2% following imbibition for 9 hours. There was still significantly higher oxidation after 9 hours in the seeds aged for one day in comparison to the high quality seeds ($P < 0.05$). When looking solely at the one day aged seeds, levels of oxidation dropped from 96.7% in dry seeds to 42.0% after 9 hours of imbibition, a decrease of 2.3-fold (Figure 6.4). Due to the large amounts of protein synthesis, increase in ROS production and other highly stressful events early in imbibition it is possible that nine hours represents a time between repair and cell cycle initiation where the genome is primed to be ready for DNA replication.

6.3.6 8-oxoG levels do not differ between actively coding and non-coding regions

The genome of *Arabidopsis* is 135 Mbp in size and can be split into regions that are actively involved in transcription (active regions; gene-coding) and those which do not and are regulatory, have other purposes or are 'junk' DNA (non-active regions; intergenic). Damage to the genome can occur across all regions of DNA; however DNA pathways can use transcription blockages as a mechanism for DNA damage detection (e.g. transcription coupled nucleotide excision repair – TC-NER). Chromatin structure also modifies around areas of high transcription, potentially affecting the presence of DNA damage and repair (Kakaroukas et al., 2015). The primers used to determine the amount of oxidation in the *Arabidopsis* genome represented a 198bp region of the *ACTIN1* gene. However, to ensure that this was representative of the genome as a whole, an untranscribed region of DNA of identical size was generated. This allows the comparison between active and non-active regions to determine whether the levels of oxidation differ dependent on genomic regional activity. DNA was extracted from seeds and treated with FPG before being amplified by PCR using primers for a coding region of *ACTIN1* and a silent region. DNA was extracted from three differently treated seeds; high quality dry seeds, dry seeds aged for 1 day and the same 1 day aged seeds that had been imbibed for 9 hours. The different treatments allows us to show that, regardless of seed quality or imbibition time point, the genome remains uniformly oxidised, with no preference for oxidation in active or non-active regions (Figure 6.5). This shows that in non-coding regions, DNA lesions are still repaired at the same frequency as those regions which are responsible for actively transcribing genes.

6.3.7 Estimating the level of oxidised nucleotides DNA in *Arabidopsis* seeds

Using primers of defined length (198bp) to determine the amount of oxidation in the *Arabidopsis* genome, and knowing the *Arabidopsis* genome is ~135 Mbp in size it is possible to extrapolate an estimate for oxidation across the entire genome. Because the levels of oxidised bases here actively coding regions do not appear to significantly differ from non-active regions (Figure 6.5), the

estimate is based on the assumption that oxidation levels do not differ significantly across different areas of the genome. Therefore, if high quality dry seeds show a 53.2% reduction in copy number then we calculate that every 198 base pairs there is a 53.2% probability of the presence of at least one oxidised guanine molecule. Extrapolating this across the whole genome (comprising ~681,000 regions of 198 bp length), this equates to 355,351 oxidised molecules in dry, high quality seeds or around 0.26% of the genome. Similarly we can do the same with the other imbibition times and ageing regimes as shown by Figure 6.4. This shows that the level of oxidation in high quality seeds drops from 355,351 oxidised nucleotides (0.26%) to 73,152 oxidised molecules (0.05%) following 9 hours imbibition. Seeds aged for one day show 639,039 oxidised nucleotides (0.47% of the genome) which drops to around 263,522 (0.19%) following 9 hours' imbibition (Figure 6.4). Investigation into the appearance of AP sites during dry storage estimates AP sites occur 38 times per 1 mbp region (Dandoy et al., 1987). This work, in *Zea mays*, would suggest an AP site would appear every ~263,000 nucleotides whereas we suggest levels of oxidation are much higher, occurring every ~380bp in high quality seeds and ~211bp in seeds aged for one day.

This approach allows us to put 'real' numbers to the developed methodology to make it more understandable and accessible however there are limitations to the extrapolation. The technique relies on assuming uniform oxidation of the genome and that each region of 198 bp in length contains only one oxidised guanine (out of 52 possible guanine molecules). However, for illustrative purposes, the prediction of actual number of oxidised guanine molecules heralds a good estimation and a useful way to present the importance of oxidation.

6.3.8 Dry *Brassica oleracea* seeds show no significant difference in DNA oxidation following maternal stress or post-harvest ageing

Arabidopsis is one of the most extensively studied model organisms due in part to its relatively simple, fully-sequenced genome and short generation time. In terms of generating biomarkers for seed quality however the methodology used must be transferable across a number of species. *Brassica oleracea* (var. *oleracea*) is an important crop species that produces orthodox seeds and has

a production output of over 70 m tonnes in 2012 (FAOSTAT, 2012). We aimed to determine whether *Brassica* seeds would respond in the same way as *Arabidopsis* to the FPG-PCR treatments as oxidation across species will potentially vary in response to maternal environment and accelerated ageing. The subjecting of crop species to unfavourable conditions leads to significant agricultural losses every year (Powell and Matthews, 2012).

The same approach was utilised with *Brassica* dry seeds, treating isolated DNA with FPG to generate single strand nicks in the DNA and then amplifying using PCR and measuring the drop in copy number after FPG treatment. Initial experiments were undertaken on dry seed, with *Arabidopsis* showing different oxidation levels after short ageing regimes (Figure 6.4). The levels of oxidation are much lower in *Brassica*, showing 15.8% reduction in high quality seeds compared to 44.4% in *Arabidopsis*. A similar trend is seen across the different maternal temperatures and ageing regimes used. It is possible that *Brassica* has more robust preventative measures in the dry seed, or potentially that seeds turnover oxidised molecules quickly by excision, resulting in more strand breaks which can be seen in section 4.2.2.3. The lack of differences in *Brassica* dry seeds suggests that the detection of oxidised bases via this method may not be suitable for use as a seed quality biomarker. Despite this, the work here in *Arabidopsis* suggests 8-oxoG has large roles to play in genomic oxidation during storage which has been shown previously (Chen et al., 2012). This may lead to development of species-specific methodology for accurately determining the contribution of oxidation to seed performance. Interspecific or intraspecific variation in repair capacities may also represent a significant factor.

Determination of base excision repair (BER) activities in seeds, in conjunction with the molecular analysis of the BER pathway in germination performance, would increase our knowledge of how genome stability influences seed quality. Various proteins have been implicated in the BER pathway in plants, including the processing of 3'-blocking ends generated by 8-oxoG incision by the protein ARP (Córdoba-Cañero et al., 2014). Genetic analysis of mutants deficient in factors, such as ARP, would establish the importance of repairing base lesions during seed storage.

7. Genome stability in ascorbate-deficient mutants

7.1 Introduction

Oxidative damage is a major cause of seed deterioration resulting from high levels of macromolecular oxidation of membrane lipids, proteins and DNA (Osborne and Boubriak, 1994; Bailly, 2004; Kranner et al., 2010; Waterworth et al., 2015). Levels of oxidative products increase with seed ageing, and reversal of this damage is important for seed longevity, as shown by the correlation between levels of the protein repair enzyme methionine sulfoxide reductase and seed longevity of *Medicago* cultivars (Châtelain et al., 2013). ROS also can have beneficial effects in cells and participate in signalling pathways, with roles in the control of germination (Bailly, 2004).

Oxidative stress is also a major cause of DNA damage. Whilst assumed to be the major cause of DNA damage in seeds, this has not directly demonstrated, although levels of the predominant form of base damage, 8-oxoG, increase after seed ageing (Chen et al., 2012; Córdoba-Cañero et al., 2014). Plants have several powerful protective systems to counter the deleterious effects of oxidative stress to the cell, which include including non-enzymatic scavenging systems, such as antioxidants tocopherol, glutathione and ascorbate, and detoxification enzymes such as catalase, ascorbate peroxidase, glutathione reductase, and superoxide dismutase (Bailly, 2004). Oxidation of seed storage proteins has also recently has been identified as a major ROS scavenging system in seeds (Nguyen et al., 2015). Collectively these mechanisms are responsible for maintaining the redox balance in plant cells.

There is a strong link between glutathione redox state and seed viability, established in several species (Kranner et al., 2006; Nagel et al., 2015). Studies investigating altered levels of glutathione in plants are difficult in seeds as mutants with reduced glutathione are not viable or display severely retarded development (Eckardt, 2007a). Analysis of ROS mutants in plants additionally can be complicated by a number of factors including high redundancy between protection systems and residual levels of antioxidants in knockout mutant lines. The study of Clerkx et al., 2004 reported that even several double mutants deficient in ROS protection systems showed only mild sensitivity to ageing and germination on H₂O₂. (Clerkx et al., 2004a).

Ascorbic acid (AsA; Vitamin C) is present in all cells of plants and has roles as an antioxidant, an enzyme co-factor and as a signalling molecule. Roles for vitamin C have been described in several processes including photosynthesis, protection from UV damage, cell growth/expansion and implications in genomic stability (Smirnoff and Wheeler, 2000; Filkowski et al., 2004). While vitamin C levels in seeds are reported to be low, a reduced antioxidant capacity during seed maturation could potentially impact on seed quality. *de novo* AsA synthesis is initiated early in germination (Smirnoff and Wheeler, 2000).

Plants completely deficient in vitamin C do not survive past the seedling stage with bleaching of the cotyledons, consistent with severe oxidative stress, and death occurring shortly after germination (Conklin et al., 2000). The major pathway for production of vitamin C in plants is the GDP-L-mannose pathway. One specific step, involving the conversion of GDP-L-galactose to L-galactose 1-P, is under the control of GDP-L-galactose phosphorylase, which encoded is by the *VTC2* gene. The *VTC2* homologue *VTC5* is semi-redundant in function, but with a minor role estimated to be around 20% of overall AsA production (Dowdle et al., 2007). Double *vtc2/vtc5* mutant are plants are non-viable; however single mutant lines contain ~15% and ~80% of wild-type vitamin C levels respectively (Figure 7.1) (Dowdle et al., 2007).

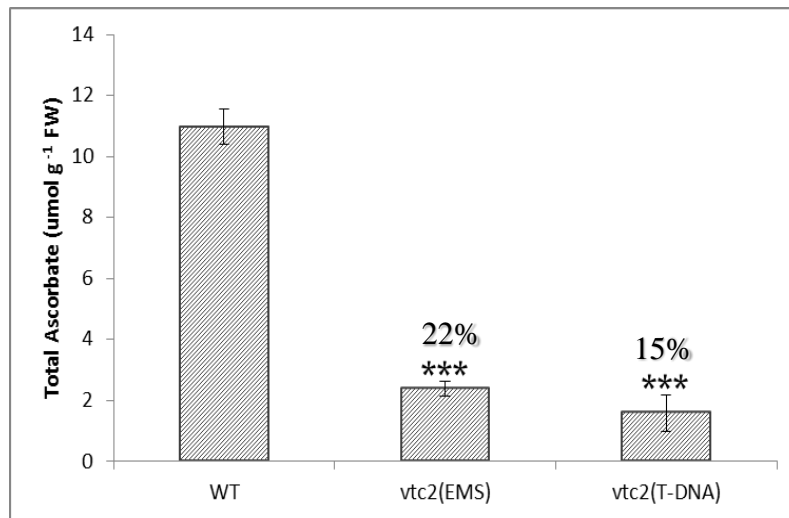


Figure 7-1 Total ascorbate levels in leaves of *VTC2* mutant seedlings (EcoSeed project; De Simone and Foyer unpublished results)

7.1.1 Aims

Oxidative damage is a major source of deterioration in seeds. One approach to minimise genome damage in seeds, and so enhance seed germination vigour and longevity, could be to increase protection of the embryo genome through increased levels of seed antioxidant levels. Here, to investigate the effects of reduced antioxidant levels on genome damage and cellular responses to DNA damage in seeds, *Arabidopsis* mutants deficient in the vitamin C biosynthesis pathway were analysed.

7.2 Results

7.2.1 Isolation and analysis of the *vtc2-5* mutant

In order to further understand the relationship between accumulation of DNA damage in seeds and ascorbic acid, two *vtc2* mutant lines were analysed. While plants cannot survive without vitamin C, it is possible to generate viable *vtc2* mutants, which have reduced levels vitamin C levels synthesised by a VTC5 dependent pathway. Two mutants lines were used in this study: *vtc2-1* is an EMS mutant previously isolated in a forward genetic screen for plants hypersensitive to ozone (Conklin et al., 1996), which contains a single base change (G to A) at the predicted 3' splice site of the fifth intron. The mutant allele results in a HindIII restriction site which can be screened using restriction analysis of a PCR generated region of the *VTC2* gene (a Cleaved Amplified Polymorphic Sequence (CAPS) marker). This mutant line was kindly donated by Professor Christine Foyer, University of Leeds. A second knockout mutant line, *vtc2-5*, was isolated as part of this study and contains a T-DNA insert (SAIL_769_H05) 620 bp downstream of the start codon. This mutation was generated through integration of the T-DNA region of the pROK2 vector through *Agrobacterium tumefaciens* infection of *Arabidopsis* plants (Alonso et al., 2003). PCR genotyping confirmed the presence of the T-DNA insertion in *vtc2-5*, utilising primers corresponding to the left border region of the T-DNA insert and *VTC2* specific primers (Figure 2.1). The wild-type *VTC2* allele was screened using primers flanking the T-DNA insertion site. No amplification across the T-DNA insertion site was observed in *vtc2-5* plants. The EMS mutant was genotyped using wild-type *VTC2* primers before analysing the amplified region for HindIII restriction sites. The wild type product of 767bp was digested to produce two smaller products of 588bp and 179bp in the *vtc2-1* mutants. PCR genotyping confirmed the presence of homozygous alleles for *vtc2-1* and *vtc2-5* (Figure 7.3). Analysis of AsA levels in seedlings was performed in the EcoSeed project by Dr Ambra De Simone and Professor Christine Foyer (University of Leeds) (unpublished data/personal communication), identifying that leaves of *vtc2-5* and *vtc2-1* seedlings respectively contain 22% and 15% of wild-type AsA levels (Figure 7.1; EcoSeed).

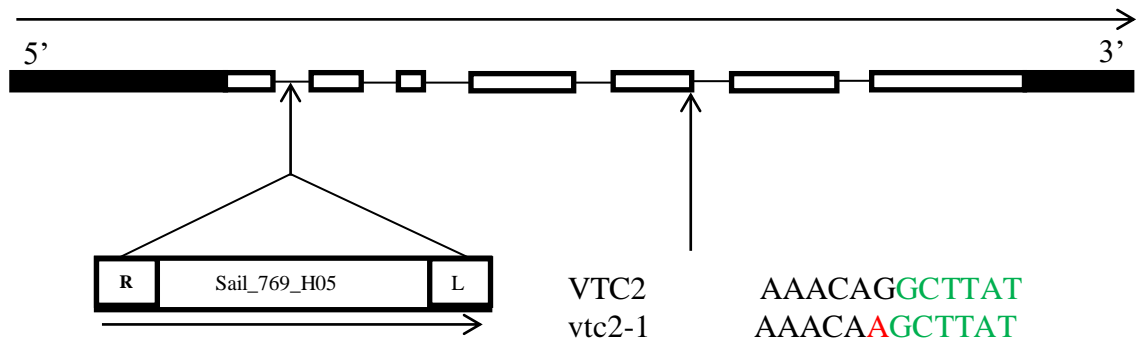


Figure 7.2 Schematic of the *VTC2* gene and location of single base change (*vtc2-1*) and T-DNA insertion (*vtc2-5*)

The position of the t-DNA insertion (*vtc2-5*) and the EMS mutation (*vtc2-1*) in the *VTC2* gene (AT4G26850). Exons are denoted by boxes, with filled boxes representing untranslated region (UTR), empty boxes representing the coded regions and introns represented by a line.

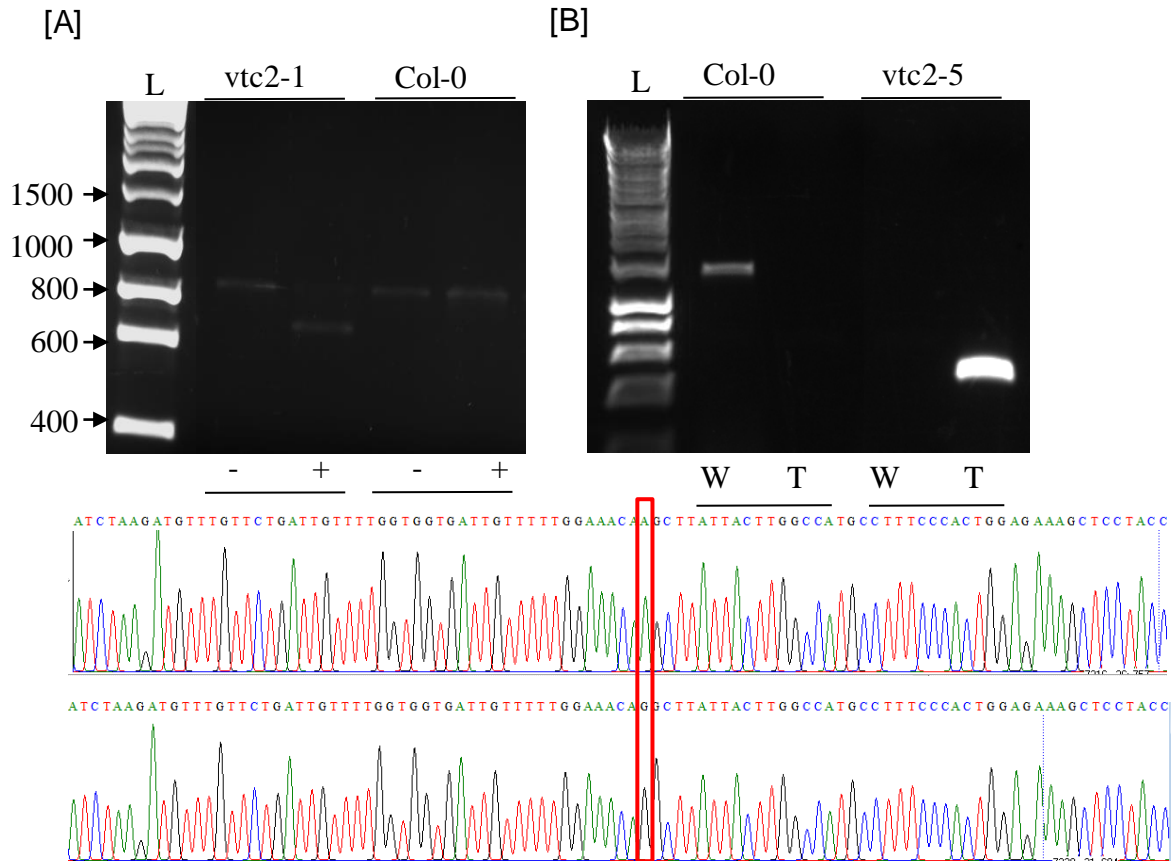


Figure 7.3 Genotyping of *vtc2-1* EMS mutant and *vtc2-5* T-DNA insertional mutant.

PCR analysis of the *vtc2-1* EMS mutant and *vtc2-5* T-DNA insertion lines. Genotyping to identify wild-type and homozygous mutant plants containing either a single nucleotide substitution (*vtc2-1*) or T-DNA insertion (*vtc2-5*). The PCR primers used are detailed in table 2.1. The ladder used was Hyperladder 1 kb plus (lanes L) with bp number shown to the left.

[A]. Wild-type (Col-0) and *vtc2-1* seedling DNA were amplified using specific primers before incubation in the absence of HINDIII (-) or in the presence of HINDIII (+). The primer pair result in an amplicon of 767bp in the absence of the mutation whereas the fragment is broken into two fragments of 588bp and 179bp following digestion in homozygous *vtc2-1* mutants.

[B] Primers specific for wild-type (W) and T-DNA insertion (T) VTC2 were used to amplify genomic DNA from plants. WT plants resulted in only a WT band and homozygous *vtc2-5* plants resulted in only the T-DNA band.

7.2.2 Germination performance of AsA mutants

AsA (Ascorbic Acid; Vitamin C) is an important plant antioxidant. Most studies into the effects of AsA on plant development to date have focused on green tissues (Clerkx et al., 2004a). Complete knockout of vitamin C in *Arabidopsis* production causes bleaching and plant death almost immediately following germination (Dowdle et al., 2007). Mutations in the *VTC2* and *VTC5* genes generate lower AsA levels in plants. Only when both *VTC2* and *VTC5* knocked out, seedlings fail to establish, with *VTC2* being the major contributor in the AsA biosynthesis pathway (Dowdle et al., 2007).

Germination performance of *vtc2-1* and *vtc2-5* mutant lines were analysed. In high quality (unaged) seed lots there were no significant difference in the final germination percentages of either mutant line when compared to the wild-type (Figure 7.4a). Both wild-type and *vtc2-5* displayed 100% final germination and *vtc2-1* a final germination percentage of 97.5%. Wild type seeds germinated within 2.00 days, *vtc2-5* seeds germinated on average after 1.98 days and *vtc2-1* seeds germinated after 2.07 days ($P > 0.1$, Students T-Test) (Figure 7.4b).

After accelerated ageing for one day (35°C; 80%) there was no significant difference in the final germination percentage of either wild-type (85.8%) or *vtc2-1* seeds (93.3%). However *vtc2-5* seed showed greater sensitivity to deterioration, with a 50.8% reduction in germination ($P < 0.01$) representing a large reduction in seed viability (Figure 7.4a). The mean time to germination also increased in wild-type (2.57 days) and *vtc2-5* (3.61 days) seeds. However, *vtc2-1* seeds did not display a significant loss of vigour (2.28 days to germination), also germinating faster than the wild-type seeds ($P < 0.01$)(Figure 7.4b).

[A]

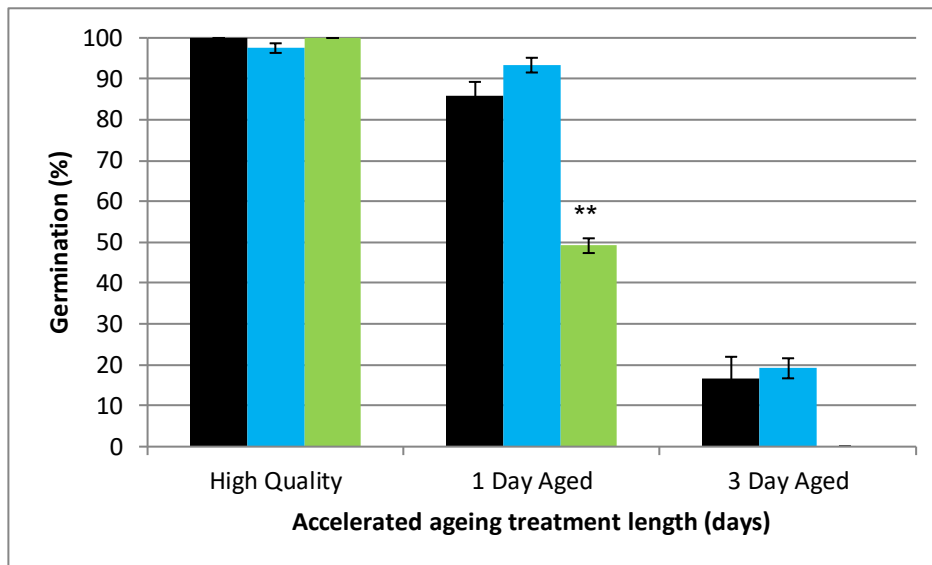
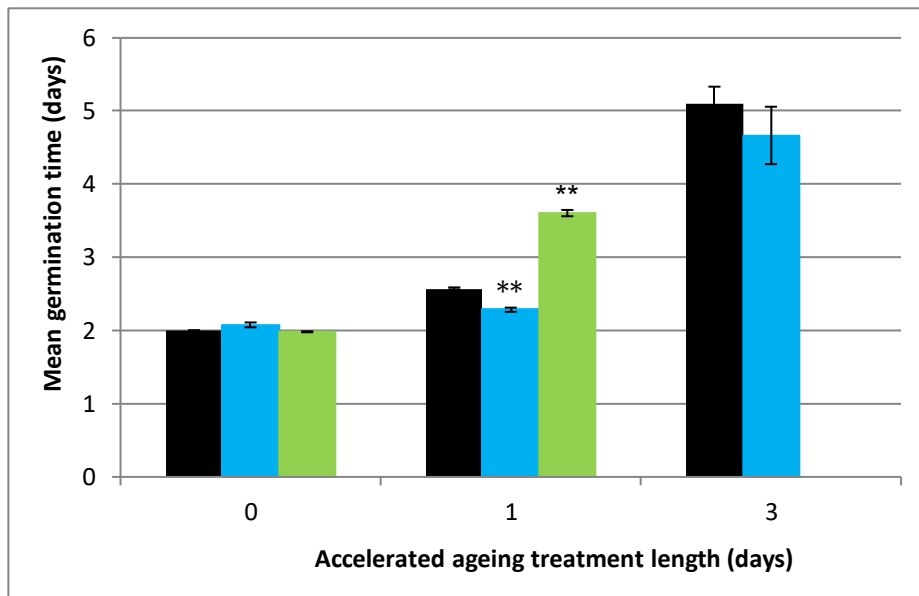


Figure 7.4 Germination performances of *vtc2-1* and *vtc2-5* mutant seed

Germination performances of wild-type, *vtc2-1* and *vtc2-5* seeds. Germination and accelerated ageing was performed as described in section 2.5.3 of wild-type, *vtc2-1* and *vtc2-5* seeds.

[A] Seed percentage viability Final germination percentages of Col-0 (■), *vtc2-1* (■) and *vtc2-5* (■) seeds after 0 days, 1 day and 3 days accelerated ageing. There was no observed difference between viability of high quality (0 days) seed lots. *vtc2-5* seeds showed reduced viability following 1 day and three days ageing, whereas *vtc2-1* germinated as wild-type seed after ageing. *vtc2-5* seeds failed to germinate following 3 days of ageing, whereas both Col-0 and *vtc2-1* seed are comparable. (error bars display SEM of 3 replicates with 40 seeds per replicate. P values: *=P<0.05, **=P<0.01)

[B]



[B] Mean germination (MGT): The mean time to germination of Col-0 (■), *vtc2-1* (■) and *vtc2-5* (■) seeds. No significant difference was observed between high quality seed lots. Following ageing for one day, germination is delayed in both Col-0 and *vtc2-5* seeds, but not *vtc2-1*. *vtc2-1* seeds germinated faster than WT seed whereas *vtc2-5* seeds took significantly longer to germinate than Col-0.

There was a large reduction in final germination percentage (viability) of all three seed lots following 3 days accelerated ageing. Whilst wild type seed displayed 16.7% germination in comparison to 19.2% of *vtc2-1* seeds, no *vtc2-5* seeds germinated, corresponding to complete loss of viability (Figure 7.4a). The wild-type and *vtc2-1* seeds displayed low germination vigour, with MGTs of 5.09 days and 4.67 days respectively (Figure 7.4b). Thus, only *vtc2-5* seed displayed significant hypersensitivity to seed ageing relative to wild-type. In contrast, *vtc2-1* seeds displayed slight improvement in germination vigour ($P < 0.01$) after 1 day of accelerated ageing. As the ageing sensitivity of the two mutants is not comparable, it is difficult to assign the observed differences in germination performed to mutation the *VTC2* gene and reduced levels of vitamin C.

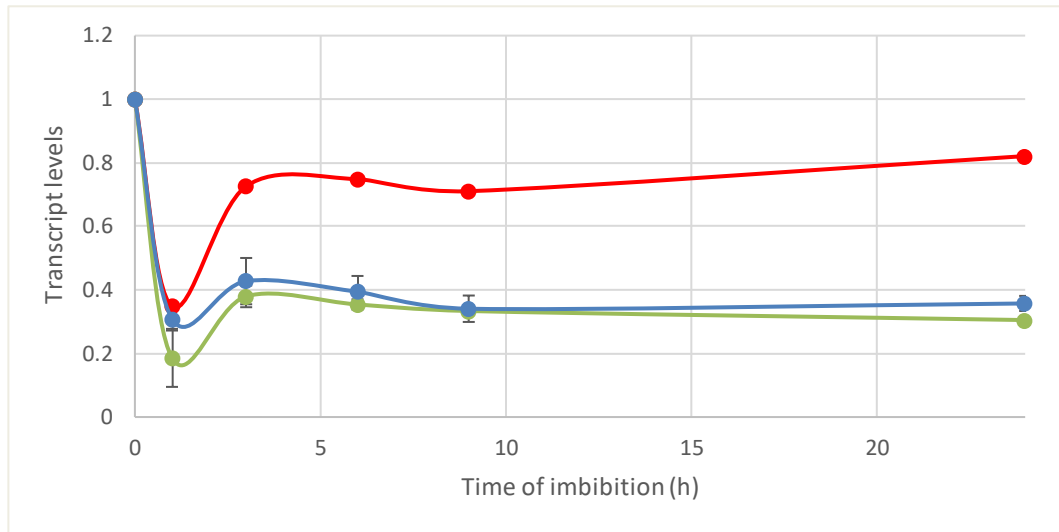
7.2.3 DNA damage associated transcriptional changes in *vtc2-1*, *vtc2-5* and wild-type seed

The roles of antioxidants such as vitamin C in protection of cellular DNA from oxidative damage in plants and influencing responses to DNA damage is poorly understood. We therefore investigated the effects of reduced levels of vitamin C in *vtc2* mutants on activation of the DDR in germination. One hypothesis was that lower antioxidant levels could lead to greater activation of the DNA damage transcriptional response as a result of increased oxidative damage to the genome in mutant seed. Transcript levels of three genes associated with the plant transcriptional DNA damage response were determined in *vtc2-1* and *vtc2-5* seeds (Figure 7.5) during imbibition of high quality (unaged) seed. Levels of transcript induction was then compared to those of wild-type seeds (Figure 7.6)

Rapid activation of the transcriptional DDR is observed early in germination of wild type *Arabidopsis* seeds. However, induction of the DDR was not observed in *vtc2-1* seeds following imbibition (Figure 7.5a), with transcript levels reducing upon imbibition and remaining low for at least 24 hours. This suggested that reduced levels of vitamin C could impair induction of the DDR. However, transcript levels in the *vtc2-5* mutant did not differ significantly from wild-type seeds (Figure 7.6), consistent with differences in germination performance of the two mutant lines. Following 6 hours imbibition, wild-type seeds showed a 21-fold induction of *RAD51* transcript levels in comparison with *vtc2-1* seed, which showed a decrease to 72% of dry seed levels. Similarly, following imbibition for 6 hours in wild type seeds *RNR* showed an 18-fold increase and *TK* a 2.5 fold increase, whereas *vtc2-1* seeds displayed a 0.35-fold reduction in transcript levels and 0.39 fold the level of the unimbibed seed (Figure 7.6). However, the reduction in DDR transcript levels observed in the *vtc2-1* mutant background was not observed in the T-DNA line *vtc2-5*, which showed transcriptional responses comparable to that of wild-type seeds. Levels of *RAD51*, *RNR* and *TK* transcripts in *vtc2-5* seed increased by 11.7, 16.0 and 3.4 fold respectively (Figure 7.6) when compared with dry seed after 6 hours imbibition. This suggests that differences seen

between *vtc2-1* and *vtc2-5* mutants are not attributable to mutations in the *VTC2* gene.

[A]



[B]

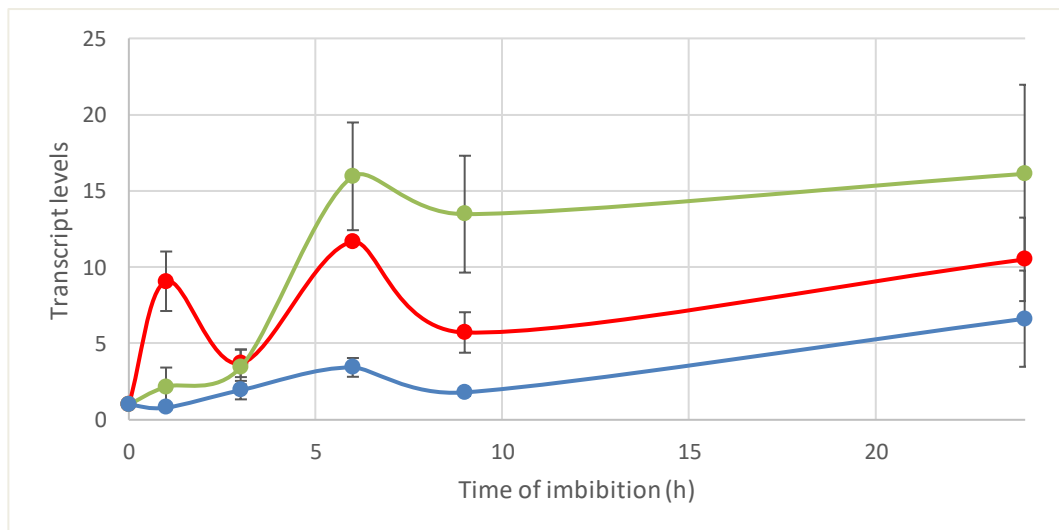


Figure 7.5 Transcriptional DNA damage response in seed imbibition of *vtc2-1* and *vtc2-5* mutants

Changes in transcript levels of the DNA response associated genes *RAD51* (■-), *RNR* (■-) and *TK* (■-) in *vtc2-1* [A] seeds and *vtc2-5* [B] seeds following imbibition for up to 24 hours. Transcript levels were measured by reverse transcription PCR (RT-PCR) and shown relative to *ACT7* transcripts. The samples were then normalised to levels found in dry seed to track changes upon imbibition. Levels of all three transcripts dropped in *vtc2-1* mutants following imbibition but the same effects were not observed in *vtc2-5* seeds with levels resembling that of wild-type with large increases seen early in imbibition (Figure 7.6).

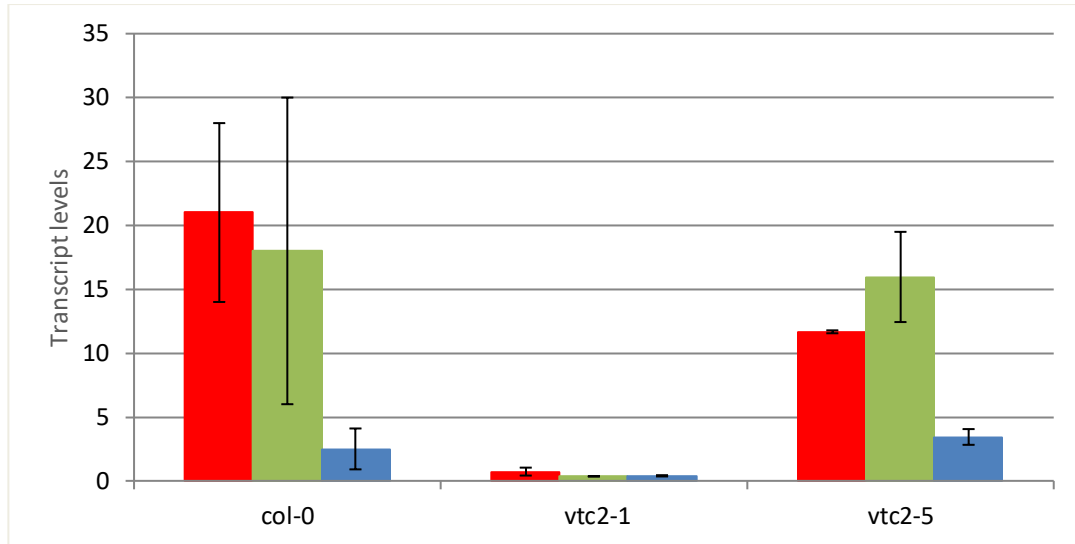


Figure 7.6 DDR transcript levels in vtc mutants following 6 hours imbibition

Changes in transcript levels of the DNA damage response associated genes *RAD51* (-■-), *RNR* (-■-) and *TK* (-■-) in wild-type (Col-0), *vtc2-1* and *vtc2-5* seed following imbibition for 6 hours. Levels of all three transcripts dropped in *vtc2-1* following imbibition, but the same effects were not observed in *vtc2-5* seeds, which did not differ from wild-type. This identifies that *vtc2-1* seeds showed decreased induction of DNA damage response associated transcripts compared with wild-type or *vtc2-5* seeds.

7.2.4 X-ray sensitivity of *vtc2-1* seeds

Double strand breaks (DSBs) cause delay to germination through activation of ATM-mediated DNA damage signalling (Waterworth et al., 2016). Evaluating plant growth following induction of double strand breaks by X-rays allows us to determine whether particular genotypes are impaired in their response to DNA damage, as root growth is hypersensitive to X-rays. Here we induced double strand breaks by subjecting seeds to varying doses (75Gy, 150Gy) of gamma radiation (Section 2.5.4). Root length was measured as a marker for reduced seedling vigour as an indicator of cell division and meristem activity (Waterworth et al., 2010). The detection of DSBs and cell cycle arrest relies on ATM, the master controller of the DNA damage response. The ATM mutant, *atm*, was also included to compare the root length in seedlings that were incapable of delaying cell cycle (Garcia et al., 2003; Waterworth et al., 2016). Wild-type seedling roots grew to 28.3 mm, whereas *vtc2-5*, showed a marked reduction in root length in relative to wild type controls (17.8 mm) ($P < 0.001$). The EMS mutant, *vtc2-1*, however displayed increased root growth compared to wild type (33.4 mm). Following 75Gy X-rays there was no significant difference observed in root length between wild-type seedlings (22.3 mm) and either *vtc2-1* (24.1 mm) or *vtc2-5* (24.4 mm) mutants. However, compared to unirradiated controls, Col-0 and *vtc2-1* displayed a similar reduction in root growth upon X-ray treatment, whereas growth of *vtc2-5* lines was not inhibited, and appeared to be stimulated by X-rays. Consistent with previous studies (Garcia et al 2003), the *atm* seeds showed a marked hypersensitivity of root growth, reflecting impaired repair of DNA damage in this mutant background. Following 150Gy irradiation the *vtc2-1* and *vtc2-5* seedlings showed some reduction in root growth (19.0 mm and 18.8 mm respectively), although only in the *vtc2-5* was this reduction statistically significant ($P < 0.01$) (Figure 7.7).

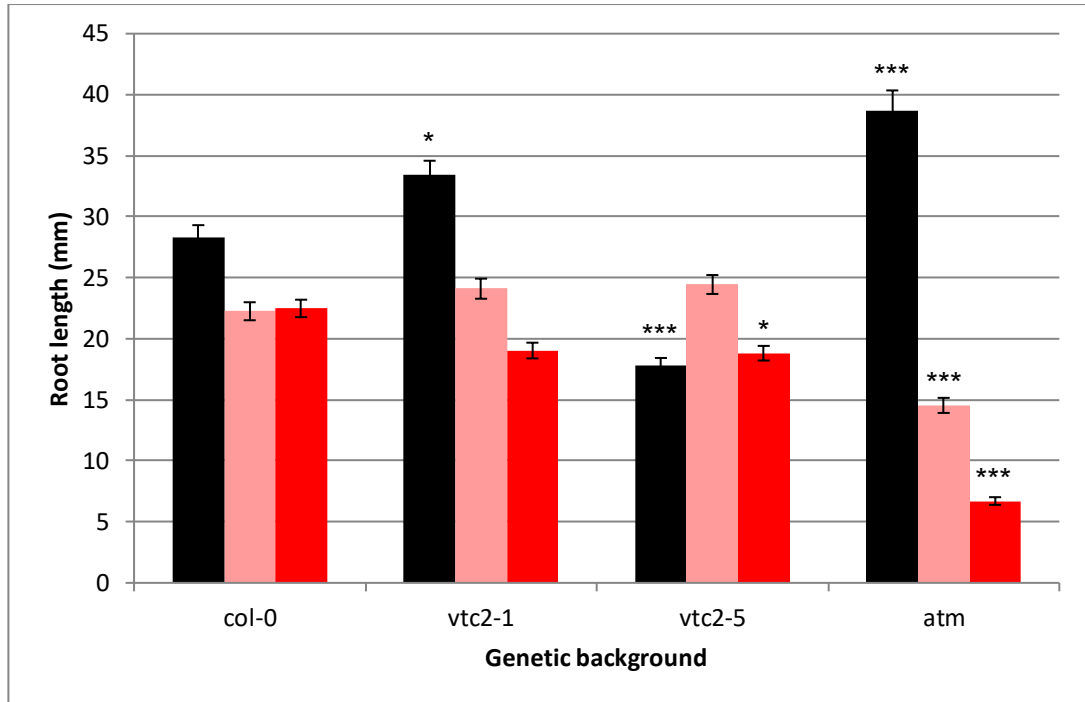


Figure 7.7 Changes in root length in wild-type, *vtc2-1*, *vtc2-5* and *atm* seedlings following gamma irradiation

Changes in root length in wild-type *Arabidopsis* and the vitamin C deficient seedlings, *vtc2-1* and *vtc2-5*, following 0Gy (■), 75Gy (■) and 150Gy (■) gamma radiation doses. Seeds deficient in the DNA damage response were also included (*atm*). Of the seeds that were not subjected to gamma irradiation, *vtc2-5* root length was decreased ($P<0.001$) whereas *vtc2-1* ($P<0.05$) and *atm* ($P<0.001$) root length increased suggesting failure to initiate normal cell cycle delay. Following 75Gy irradiation there was no difference in either *vtc2* mutant but *atm* seeds showed hypersensitivity of root growth ($P<0.001$). Only *atm* ($P<0.001$) and *vtc2-5* ($P<0.05$) seedlings showed reduced root length following 150Gy irradiation. All statistical analysis is a result of student's t-test with a minimum of 25 samples per condition (P values: *= $P<0.05$, **= $P<0.01$, ***= $P<0.001$, in comparison to identically irradiated Col-0 samples).

7.2.5 Oxidised guanine accumulation in vitamin C deficient seeds

A role of vitamin C in protecting the embryo genome from oxidative damage was investigated by determination of the amount of oxidised guanine base damage present in wild-type seeds and in seeds deficient in vitamin c (*vtc2-1*) (Figure 7.8) throughout imbibition. Genomic DNA was isolated from dry seeds (0 hours imbibition) or seeds imbibed for 3, 6, 9 or 16 hours. These seeds were then analysed for 8-oxoG accumulation using FPG treatment and PCR quantification as described in section 6.2.

Despite the lower levels of vitamin C in the mutant, there were no significant differences in the levels of oxidised base damage observed at the time points analysed during seed imbibition. Dry Col-0 seeds showed 8-oxoG base damage in 38.2% of copies of the genomic region analysed. Vitamin C-deficient seeds accumulated damage to 53.1% of loci, although this was not statistically different from levels measured in wild type seeds. At an early imbibition time point of 3 hours, wild-type and vitamin C-deficient seeds displayed 52.2% and 59.9% loci respectively containing damage. This did not change at the 6 hour imbibition time point, with levels measured as 45.9% and 52.8% damaged loci in wild type and *vtc2-1* seeds respectively. 8-oxoG levels were quantified at the 16 hour imbibition time point, but no significant differences were observed between wild-type seeds (47.8% damaged loci) and *vtc2-1* seeds (52.2% loci with damage).

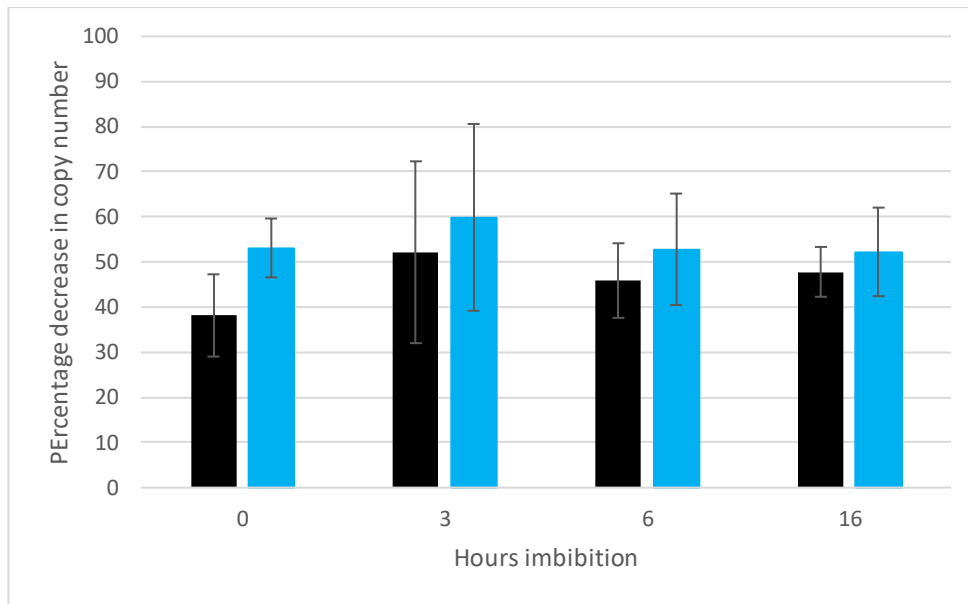


Figure 7.8 Accumulation of oxidised guanine molecules in wild-type and vitamin C deficient seeds during imbibition

8-oxoG accumulation was determined in wild-type *Arabidopsis* (■) and *vtc2-1* seeds (■) during imbibition. No differences in accumulation of 8-oxoG were observed in dry seed (0hours), 3 hours, 6 hours or 16 hour imbibed seeds.

7.3 Discussion

7.3.1 Differences in germination capacity of vitamin C deficient seeds

Vitamin C is ubiquitous in plant tissues and is found in all plant species to date (Wheeler and Smirnoff 2000). Despite the importance of vitamin C in plant tissues, its functions in plants remain to be fully elucidated. A relationship between the antioxidants tocopherol and glutathione and seed deterioration is established (Sattler et al., 2004; Kranner et al., 2006; Nagel et al., 2015). Ascorbic acid synthesis occurs as the seed matures on the mother plant, but levels drop during maturation drying, although small amounts of dehydroascorbic acid (DHA) and large amounts of vitamin C recycling enzymes remain present (De Tullio and Arrigoni, 2003). This suggests vitamin C may have a minor role in protection from ROS in dry seed during storage but may influence the oxidation state of the dry seed due to roles during the maturation phase. Significantly, overexpression of antioxidant genes in *Arabidopsis* conveys increased resistance to oxidative stress early in seedling growth (Xi et al., 2010). Illustrating that the complex interactions and high redundancy of ROS protection mechanisms often complicates their analysis in plants, stress resistance in this study was only conferred by combination of pathways. The synthesis of vitamin C begins within a few hours of imbibition, consistent with roles in protecting the seed from oxidative damage associated with the loss in subcellular compartmentation that occurs during the early stages of rehydration (Sreenivasan and Wandrekar, 1950). However, synthesis would be dependent on the reinitiation of metabolism in the seed. Early imbibition is important as repair processes are initiated at this time, representing critical events in prior to germination that support successful seedling establishment (Elder and Osborne, 1993; Waterworth et al., 2016). The antioxidant levels in the dry seed may therefore be crucial in the earliest stages of imbibition, when metabolism has not commenced but the re-entry of water generates high levels of oxidative stress. This provides the potential for vitamin C to influence seed vigour, and a possible relationship between vitamin C levels and genome stability has been established (Filkowski et al., 2004). Direct interaction with DNA was demonstrated at millimolar concentrations of

vitamin C where it bound to duplex DNA, although the physiological relevance of this observation is unclear (Yoshikawa et al., 2006).

Vitamin C levels in the two *vtc2* mutant lines were similar, at around 22% and 15% of wild-type levels in *vtc2-1* and *vtc2-5* seedlings respectively (Figure 7.1), although levels in the dry seed and early imbibition were not determined. Germination performance of *vtc2* mutant seed was analysed. In high quality (unaged) seeds there was no difference in seed vigour or viability, between the mutant and wild type lines. Lower levels of the antioxidant in mutant seed does not affect germinate under normal conditions; however 1 day ageing reduces vigour but not viability in Col-0 seeds, whereas the *vtc2* mutant lines displayed different responses. *vtc2-5* seeds took much longer to germinate and final germination percentage was reduced to below 50%, consistent with reduced ROS scavenging during imbibition in the mutant. However *vtc2-1* seeds did not display this phenotype. Despite similar levels of ascorbate, *vtc2-1* seeds germinated slightly faster than wild-type seeds, with vigour levels unaffected by 1 day accelerated ageing. The hypersensitivity of *vtc2-5* to ageing led to significantly reduced viability and vigour following ageing, whilst *vtc2-1* seeds displayed similar or higher germination levels as wild-type. The different response observed in the two mutant background could reflect the effects of mutation either in different regions of the *VTC2* gene, or genetic differences in the lines unrelated to the *VTC2* gene. The *vtc2-1* seeds were generated through EMS mutagenesis followed by out crossing of background mutations, whilst the *vtc2-5* line was generated through T-DNA insertion into the *VTC2* gene, selected from a large population of random insertion lines (Sessions et al., 2002).

The combination of the increased seed vigour of *vtc2-1* seeds (Figure 7.4) and the negligible induction of the transcriptional DDR during imbibition (Figure 7.5A) might support the conclusion that an increased oxidative state of these seeds resulted in faster germination. mRNA oxidation has been shown to influence germination of rice seeds and promote releases from dormancy in sunflower (Bazin et al., 2011). Although *vtc2-1* seeds germinated faster they also displayed signs of bleaching in seedlings, possibly resulting from an apoptosis-like mechanism (Lim et al., 2016).

7.3.2 Effects of reduced levels of vitamin C on the transcriptional DDR

Transcript levels of the DNA damage response associated genes *RAD51*, *RNR* and *TK* were analysed in vitamin C deficient mutants to determine whether vitamin C levels could influence the transcriptional DNA damage response. Plants initiate synthesis of new vitamin C early in imbibition, around the same time that transcriptional DNA damage response (DDR) is activated (Sreenivasan and Wandrekar, 1950; Waterworth et al., 2010). Vitamin C levels have been reported to influence transcription of a variety of gene families. The majority of transcripts modulated by vitamin C are associated with biotic defence; of the 171 genes found to be influenced by ascorbate levels, 33 were found to be involved in cell cycle control or have DNA binding capacities, with a further 11 involved in protein synthesis and protein modification (Pastori et al., 2003). All changes in transcripts reverted to wild-type levels when *vtc2* mutant lines were supplemented with ascorbate (Pastori et al., 2003).

The effect on transcripts could also be modified by changes in ROS levels of the seeds, which would be affected by the reduced antioxidant levels in the *vtc* mutant lines. The presence of elevated levels of oxidative species initiates a cascade of signalling pathways, resulting in transcriptional responses associated with cell rescue and defence (Maurino and Flügge, 2008). Furthermore mRNA is prone to oxidation in high oxidative state environments due to its single stranded nature and lack of repair mechanisms. Damaged mRNA is turned over in the cell, and while the mechanisms for quality control remain obscure, the physiological importance is illustrated by the requirement of oxidation of mRNA in sunflower in order to alleviate dormancy (Bazin et al., 2011). Should the *vtc2-1* seeds have vitamin C levels low enough to enhance cellular oxidation, particularly of mRNA molecules, it may induce early germination as seen in Figure 7.4b.

Because of the differences observed in germination performance and transcript levels in the two *vtc2* mutants, it is difficult to draw firm conclusions regarding the observation that altered transcript levels are observed only in *vtc2-1* (Figures 7.5a and 7.6). *vtc2-5* seeds showed no difference from wild-type, although plants contain around 15% of the ascorbic acid content. This suggests that ascorbate levels do not affect the DDR mediated transcriptional

induction, either directly or through differences in ROS levels. Thus, the phenotypic effects of *vtc2-1* on the DDR are likely to be independent of ascorbate levels, and could be related to background EMS induced mutations genetically linked to the *VTC* locus that were not removed in out-crossing

7.3.3 Sensitivity of *vtc* mutants to DNA damage

In *Arabidopsis*, wild type levels of germination vigour require activation of the cell cycle prior to germination (Masubelele et al., 2005). The presence of DNA damage arrests to cell cycle progression to reduce the mutagenic effects of DNA damage (Waterworth et al., 2016). Delayed meristem activity results in reduced root growth following irradiation (Liu et al., 2004; Fulcher and Sablowski, 2009). Root length was quantified in wild-type, the vitamin C deficient *vtc2-1* and *vtc2-5* seeds along with *atm* seeds to determine whether the *vtc2* mutants displayed hypersensitivity to DNA damage.

In addition, wild type and mutant seeds were subject to X –ray irradiation to determine the effect of vitamin C (and antioxidant levels) on the cellular response to DNA double strand breaks (Plumb et al., 1999; West et al., 2000). Vitamin C has been shown to provide dose-dependent protection of DNA in the presence of double strand breaks with 5 mM treatment having 20 times lower levels of DSBs (Yoshikawa et al., 2006). We found that root length was reduced in *vtc2-5* seeds relative to wild type in the absence of irradiation but both *vtc2-1* and *atm* displayed longer roots than controls (Figure 7.7). Following irradiation root length in *atm* seeds was reduced significantly relative to WT, the typical phenotype of NHEJ knockout mutants in *Arabidopsis*, with accumulated DNA damage, lack of root cell maintenance and loss of dividing cells (Garcia et al., 2003; Ricaud et al., 2007). In *vtc2-5* seeds treated with 150Gy X-rays there was a significant reduction in root length, typical of cell cycle arrest in the presence of increased DNA damage. Inhibition of root growth has been reported to be reduced in the presence of enhanced oxidative stress by exogenous application of vitamin C, showing the importance of the antioxidant in root meristem cells early in germination (Xu et al., 2015). Unusually the same seeds subjected to 75Gy radiation had longer roots than those at 150Gy suggesting increased protection following mild irradiation treatments; however X-ray doses above 100Gy has been shown to cause

variation in levels of vitamin C in onion seeds which could account for the shorter roots (Benkeblia and Khali, 1996)

7.3.4 Genomic protection from reactive oxidative species is not dependent on vitamin C in seeds

To determine whether the reduction in vitamin C levels, and thus the reduction in ROS scavenging, would affect the oxidation of the genome, levels of oxidised guanine molecules were quantified throughout seed imbibition. Conversely, it has been shown that loading human cells with dehydroascorbic acid reduces mutation frequency and accumulation of oxidised guanine molecules induced by H₂O₂ (Lutsenko et al., 2002). Despite the preventative nature vitamin C has on reactive oxygen species induced damage, it also has pro-oxidant properties when in the presence of free transition metals. Several transition metal-requiring enzymes are present in soybeans during seed growth including iron, copper and zinc dependent enzymes, leaving the opportunity for oxidised ascorbate to potentially generate oxidised base lesions (Agrawal et al., 2008). There was no significant difference observed in levels of 8-oxoG between the *vtc2-1* seeds and wild type, indicating that reduction of vitamin C did not significantly affect genomic oxidation levels. Although oxidised guanine is commonly used as a marker of oxidative damage, and studies report lower levels following vitamin C supplementation (Podmore et al., 1998; Lutsenko et al., 2002) there is also evidence that the pro-oxidant nature of oxidised vitamin C favours production of 8-oxo-adenine over 8-oxo-guanine (Podmore et al., 1998). This would suggest that lower vitamin C levels in the *vtc2-1* mutant could potentially have reduced levels of genomic oxidation to adenine molecules in early imbibition which would account for some of the resilience to ageing seen in Figure 7.4. However due to the prevalence of oxidised guanine molecules in comparison to the relatively scarce levels of oxidised adenine measuring levels of 8-oxo-adenine makes quantification difficult and not as relevant in seed genomic oxidation (Cadet et al., 2003).

In mammals and yeast the also DDR is known to be activated directly in response to oxidative stress and ATM is well established to functioning as a major redox sensor. However, in plants such a role remains obscure to date.

Two lines of evidence support a link between DDR induction and redox sensing in plants. Double *cat/asp* mutants deficient in ascorbate peroxidase and catalase, but not the single genes, displayed constitutive elevated expression of genes associated with the DDR, were resistant to genotoxic stress and induced expression of cell cycle inhibitor WEE1 (Vanderauwera et al., 2011). However, DDR induction levels were low and the gene profile was distinct from that of the DDR profile observed in seeds to genotoxic stress e.g. BRACA1 was up regulated. That single mutants did not demonstrate this response suggested that synergistic interaction of two pathways is required for activation of the DDR by ROS. Furthermore, the cell cycle inhibitors SMR5 and 7 are transcriptionally induced by oxidative stress inducing stimuli under control of ATM (Yi et al., 2014). The authors suggest that, as SMR5 is transcriptionally induced by a wide range of stresses, that SMR5 could function to integrate ROS signalling/redox status with cell cycle control

The DDR is activated very early in seed imbibition, indicative of DNA damage sensing by ATM, in response to DNA damage. SMR5 is also major component of the mechanism which integrates control germination with ATM (Waterworth et al., 2016). An intriguing idea is that redox status in the germinating seed could directly contribute to regulation of germination through ATM and SMR5.

8. General Discussion

8

General discussion

Seed quality is a major determinant of crop yield and underpins effectiveness of conservation programmes in seedbanks, yet the molecular aspects governing the change in germination vigour and overall viability are far from understood (Finch-Savage and Bassel, 2016). From the early stages of development, through desiccation acquisition, quiescence and imbibition, seeds undergo a series of abiotic stresses that can impact on seed quality. Understanding how environmental and endogenous stresses affect seeds allows the prediction and enhancement of seed longevity and will help in the assessment of the potential impact of changing climates on crop performance. Molecular changes that correlate with germination performance have potential as new biomarkers for seed quality, which are required to be relatively simple, cost-effective and applicable to a range of species. The present study focussed on lesions in DNA in seeds grown and stored under different environmental conditions, including an analysis of the DNA damage responses that occur early in seed imbibition and the correlation with germination performance.

8.1 DNA integrity in seeds

8.1.1 Single strand breaks are more frequent in aged seeds and levels increase upon imbibition

DNA damage accumulates in the seed as a result of stresses during maturation, desiccation, storage and imbibition. Single strand breaks in DNA arise through both damage to the sugar phosphate backbone and through excision of damaged bases in BER mechanisms. The levels of apyrimidinic (AP) sites in dry *Zea mays* seeds has been estimated at 38 sites per 10^6 bases following two years of storage, which increased four-fold following imbibition (Dandoy et al., 1987). The quality of the seeds used, in terms of germination percentage and vigour, is unclear although it is suggested the seeds are capable of germinating within 36 hours. The work presented in this thesis aimed to correlate seed germination performance with SSB levels in unaged and aged seeds produced from plants grown under different temperature stresses. Maternal environment temperature ranging from 15-29°C had no significant effects on the number of single strand breaks present in either *Arabidopsis* or *Brassica oleracea* seeds, whether dry or imbibed (Figures 4.4 – 4.7) in this study. Previous work revealed that both elevated and reduced temperatures led to increased genome instability, as measured by increases in spontaneous chromosomal rearrangements (Boyko et al., 2005). However, any destabilisation of the genome was either not carried over to seeds, or was not detectable with the approaches used in the present study. However, a significant accumulation of SSBs, coinciding with a reduction in viability, was observed aged *Brassica* seeds (Figures 4.6 and 4.7). Elevated levels of SSBs in aged seed persisted from the dry seed, through early imbibition (end of phase I germination) and in to late imbibition in aged *Brassica* seeds. In line with the increase in AP site observed upon imbibition of maize (Dandoy et al., 1984), SSB levels also increased over two-fold in *Brassica* following imbibition.

8.1.2 Single strand breaks as a potential biomarker for seed quality

The correlation between single strand break accumulation and seed quality has potential to be utilised as a biomarker to determine or predict the quality of seed lots. Prediction of seed lot quality underpins both the analysis of seed

lots in the seed industry and is important to the conservation of plant genetic resources in seedbanks. There is currently an acute demand for universal, quantitative and sensitive biomarkers to replace manual germination testing. The increase in SSBs observed in both *Arabidopsis* and *Brassica* accompanied significant loss in viability. In the seed industry, seed batches are required to be of high viability, of over 90% final germination percentage. Vigour differences between high viability commercial seed lots are the main determinant of crop productivity losses, with low vigour seeds leading to poor seedling establishment (Finch-Savage and Bassel, 2015b). The SSB detection methods here lacked sufficient sensitivity to identify differences in damage levels in seed lots of different vigour before loss of viability. However, predictors of viability loss are required for routine evaluation of seed lots stored in seedbanks, and quantification of DNA integrity could provide a universally applicable and widely accessible approach for utilisation in seed conservation programs.

The increase in SSB levels observed on imbibition may reflect the activity of excision repair pathways, or *de novo* generation of breaks through oxidative stress arising from the influx of water. A second possible source of damage could be release of ROS through deterioration of subcellular compartmentalisation in deteriorated seed. If the former proved true it would indicate that cells undergoing vast amounts of repair, rather than passively accumulating damage. Analysis of excision repair deficient mutant lines would help distinguish which of these two hypotheses is correct, as reduced repair capacity would lead to fewer SSBs generated after imbibition.

8.2 DNA damage responses in imbibition

8.2.1 Temporal patterns of the DNA damage transcriptional response

The DNA damage response (DDR), mediated by ATM, is highly specific to increased levels of double strand breaks (DSBs) and activates the transcriptional induction of hundreds of repair-related genes (Molinier et al., 2005; Culligan et al., 2006; Waterworth et al., 2010). The DDR is activated early in imbibition of *Arabidopsis* seeds, reflecting a rapid response to

genotoxic stress encountered throughout storage and upon imbibition, both of which are associated with high levels of oxidative stress (Bailly, 2004; Kranner et al., 2010). The magnitude of the transcriptional response reflects levels of DNA damage, with greater amounts of DSBs causing larger transcriptional induction. Here the aim was to determine the magnitude of the DDR in seeds of different quality using a reverse transcription-PCR based method to indirectly correlate levels of DNA damage with loss of seed viability.

8.2.2 Temporal patterns of *RAD51* transcripts in seeds of different quality

RAD51 is an essential gene in *Arabidopsis* which mediates strand invasion during repair of double strand breaks utilising the homologous recombination (HR) repair pathway. The level of *RAD51* transcripts increases over 100-fold following 100Gy γ -ray irradiation (Culligan et al., 2006). Presence of *RAD51* transcripts has been shown to increase significantly early in imbibition of *Arabidopsis* and barley (Waterworth et al., 2010; Waterworth et al., 2016). Consistent with this previous report, the present study found that transcript levels increased up to 14-fold following 6 hours imbibition (Figure 5.1). The increase in HR-related proteins is unusual in early imbibition, particularly because the pathway is rarely used in higher plants in comparison to error-prone NHEJ outside of replicating cells. Environmental temperature during seed development on the mother plant did not affect levels of *RAD51* in dry seeds or throughout imbibition in this study. However, ageing did have a significant effect. Controlled deterioration significantly attenuated the transcriptional DNA damage response, with little transcript induction at 6h imbibition in deteriorated seeds, contrasting with the rapid upregulation of *RAD51* observed in high quality seeds at this time point. This response was unexpected because seeds subjected to ageing accumulate DNA breaks (Waterworth et al., 2015, 2016). The attenuated transcriptional response is therefore highly unlikely to represent a reduced need for repair activities in aged seeds. Rather, the weakened DDR may result from cellular damage and reduced transcriptional capacity of the aged seed, possibly arising from low cellular energy levels and/ or macromolecular damage (Kranner, 2010). Consistent with this hypothesis, ageing resulted in a very large reduction in

cellular translation activity in *Arabidopsis* seeds (Rajjou et al., 2008). Alternatively, oxidation-induced RNA damage may lead to transcript turnover (El-Maarouf-Bouteau et al., 2013). Interestingly, the attenuated DDR upon ageing is observed in seeds that display reduced vigour but retain high viability (Figure 3.2). This suggests that dampening of the transcriptional response is a very early symptom of seed ageing.

8.2.3 Temporal patterns of *RNR* transcripts in seeds of different quality

Ribonucleotide reductase (*RNR*) is important in synthesis of the dNTPs required for DNA repair and replication processes. Following 100Gy γ -ray irradiation (Culligan et al., 2006), the level of *RNR* transcripts increased 37-fold as part of the transcriptional DDR. As observed for *RAD51*, *RNR* transcripts were induced upon imbibition, although delayed relative to *RAD51*, peaking around 72 hours (Figure 5.2). Again no differences in *RNR* transcript levels were associated with different environmental conditions during seed development on the maternal plant. This transcriptional induction of *RNR* was again largely absent following controlled deterioration of seeds, most likely linked to increased damage to transcriptional machinery arising from elevated ROS levels in seed ageing (Coello and Vázquez-Ramos, 1996). The reduced magnitude of the DDR may increase the time taken to repair cellular damage and this could be a major contributing factor to the extended time taken for aged seeds to germinate. It would be informative to determine levels of repair proteins and activities of repair pathways in aged seeds, and the extent to which these limit germination and seed vigour.

8.2.4 Transcript abundance as a biomarker for seed quality

The DNA damage response (DDR) initiates signalling and activates pathways involved in maintaining genome integrity and cell cycle control. In plants our understanding of the effects of downstream signalling in the DDR, mediated by ATM, is incomplete, with most studies limited to transcriptional responses and, more recently, phosphoproteomics analysis (Roitinger et al., 2015). Phosphorylation of histone H2AX is highly conserved across eukaryotes and is used extensively as biomarker for double strand breaks (Kuo and Yang, 2008). H2AX phosphorylation is well-characterised in plants, but detection in

seeds has not been reported to-date. An original aim of this project was to investigate the ATM-dependent DDR (Waterworth et al., 2015, 2016) as an indirect method to determine elevated levels of DSBs in aged seeds, reported by increased transcriptional induction of *RAD51* on imbibition. However, in contrast to the expected result, an attenuated DDR in lower quality seeds was observed, even after mild ageing treatments that lead to a reduction in seed vigour but maintained high viability. Thus there is potential for the attenuated DDR as a marker for loss of seed vigour, if similar observations can be made in other species.

8.3 The molecular impact of oxidation in seeds

8.3.1 Combined effects of maternal environment and seed ageing on 8oxoG abundance

Oxidative DNA lesions, specifically 8oxoG, are one of the most common genomic lesions incurred by seeds due to large amounts of oxidative stress associated with desiccation, storage and imbibition. The lesions are removed by either FPG or OGG1 DNA glycosylases in plants via the base excision repair (BER) pathway, with either gene sufficient for removal of this lesion. One difference between the two enzymes is the ability for FPG to generate 3'-P termini that are not evident in mutant *fpg Arabidopsis* plants but are present in *ogg1* lines (Córdoba-Cañero et al., 2014). 8oxoG lesions were quantified during seed imbibition to determine when greatest oxidative damage was incurred and repaired in seeds. Oxidation of macromolecules is a major feature of seed deterioration and shown to be an underlying cause for the loss in viability during storage (Bailly, 2004; Kranner et al., 2010). Previous studies demonstrated increased that 8-oxoG levels increased following seed imbibition but were reduced upon overexpression of the BER factor OGG1, which increased seed longevity and resistance to ageing (Chen *et al.*, 2012). During BER removal of 8oxoG, zinc finger DNA 3'-phosphoesterase (ZDP) and apurinic endonuclease redox protein (ARP) are involved in post excision steps and knock-out mutants in these two lines show increased sensitivity to seed ageing (Córdoba-Cañero et al., 2014). Similarly, we found increased oxidation of the *Arabidopsis* genome following imbibition, but surprisingly levels were not increased in aged seeds. The exception was seeds from plants

grown in lower temperature environments where, upon controlled deterioration, an increase in oxidation was seen in the dry seed (Figure 6.4). These seed lots also failed to show increased oxidation upon imbibition, perhaps reflecting delayed activation of metabolic processes in these low vigour seeds.

8.3.2 Fluctuations in oxidative damage during imbibition

Seeds produced under differing maternal temperature regimes and subjected to controlled deterioration showed no consistent patterns in levels of genome damage, either in response to treatments or during imbibition. However, the variation observed in 8oxoG levels was indicative of active oxidation and 8oxG removal by genome repair pathways. Unaged, high viability seeds displayed 40% less oxidative damage than seeds aged for 1 day (Figure 3.1), indicative of significant genomic oxidation during ageing. Interestingly, repair kinetics seemed similar between aged and unaged seeds, with lowest 8oxoG levels observed at 9 hours post imbibition, which is around 3 h after the peak of DNA damage response gene induction (Figure 7.6) (Waterworth et al., 2010). Levels of oxidation increase again towards the end point of germination, which may relate to ROS-signalling previously reported in seeds (Bailey, 2004).

8.3.3 Repair of genome damage at different loci

Transcription-coupled nucleotide excision repair (TC-NER) preferentially repairs active genes by detecting stalling of the RNA polymerase complex at a transcription-blocking lesion and activating repair processes. Quantification of 8oxoG levels in the actively transcribed *ACTIN1* gene and an intergenic region revealed no differences in either oxidation or repair between coding and non-coding regions. This therefore suggests that oxidative DNA repair is a global process and not confined to protein-coding regions, and may also reflect the fact the 8-oxoG does not block transcription and so would represent a poor substrate for TC-NER.

8.3.4 Levels of oxidative DNA damage as a biomarker for seed quality

Increased levels of base oxidation were observed upon *Arabidopsis* seed imbibition, which reduced by 9 hours imbibition, possibly reflecting activity of repair processes, and increasing as the seed neared germination. However,

base oxidation levels in *Brassica* seeds did not show similar trends in response to environmental temperature either during seed development or after controlled deterioration (Figure 6.7). Thus, key trends in genomic oxidation using the seed lots analysed in the study were not clearly identified. Further work is required to determine how levels of oxidative stress and damage fluctuate during seed imbibition. The seed stage of the plant lifecycle is associated with extremely high levels of genotoxic stress in the orthodox seeds not encountered nor tolerated under physiological growth conditions (Waterworth *et al.* 2010; 2012; 2015; 2016). Thus, the orthodox seed has consequently evolved a remarkable tolerance to DNA damage, so to what extent variation in damage levels between seed lots is attributable to programmed events in germination, or incidental to stresses that reduce seed germination potential remains to be established.

8.4 Antioxidant levels and genome integrity

8.4.1 Vitamin C levels are tightly controlled

To further investigate the relationship between oxidative stress and genome damage in the orthodox seed, we used *VTC2* mutants deficient in vitamin C synthesis. Low molecular weight antioxidants in plants include glutathione, tocopherol and vitamin C (ascorbate, AsA). Glutathione redox status correlates with germination potential in several species (Kranner *et al.*, 2006; Nagel *et al.*, 2015). However, mutants with reduced glutathione are often non-viable or display severely retarded growth, compromising analyses in these mutant backgrounds. However, *Arabidopsis vtc2* knockout mutants deficient in synthesis of the antioxidant vitamin C are viable, as they retain residual low levels of AsA arising from the activity of *VTC5*, which provides a minor pathway for AsA synthesis. Thus *vtc2* mutants produce around one-fifth the levels of vitamin C observed in wild type lines in leaves of young seedlings (Figure 7.1). The L-galactose pathway is responsible for most AsA synthesis and mutation disrupting this pathway results in lethality early in seedling growth (Dowdle *et al.*, 2007). Thus vitamin C is not required for the completion of germination but is required post-germination. Conversely, plants with enhanced vitamin C levels display increased fresh weight, longer roots, improved H₂O₂ scavenging and improved germination rates in some species, but decreased germination

vigour in wheat (*Triticum aestivum*) (De Gara et al., 1991; De Gara et al., 2000; Ishibashi and Iwaya-Inoue, 2006; Behairy et al., 2012).

8.4.2 Germination characteristics of vitamin C-deficient mutants

Vitamin C deficient mutants were used in the present study to investigate the relationship between altered antioxidant levels on accumulation of DNA damage and resistance to seed ageing. Surprisingly, the germination performance of the *vtc2-1* and *vtc2-5* mutants analysed in the present study differed, with *vtc2-1* (EMS generated point mutation) displaying higher vigour than wild-type following seed ageing, and *vtc2-5* (T-DNA insertional mutant line) displaying reduced viability and vigour upon accelerated ageing. These differences between the EMS and T-DNA mutants must be attributable either to the differences in the mutation in the *VTC2* gene or differences between the two genotypes elsewhere in their genomes. The latter is a more likely with recent studies suggesting the growth retardation in *vtc2-1* plants (not seen in *vtc2-5*) segregates independently of the *vtc2-1* mutation (Lim et al., 2016). The *vtc2-1* mutation therefore evidently carries background mutations in one or more unknown genes.

8.4.3 Activation of the transcriptional DDR in *vtc2* mutants

Induction of the genes associated with the transcriptional DNA damage response (DDR) was also different in the *vtc-2* mutant background. Whereas *vtc2-1* seeds showed no activation of the transcriptional DDR in imbibition, *vtc2-5* seeds displayed an activation of the DDR early in germination comparable to that observed in wild-type (Figures 7.5-7.6). This further disparity between the two *vtc2* mutants strongly supports the effects of background mutation in the *vtc2-1* line (Lim *et al*., 2016). The complete lack of DDR induction in *vtc2-1* also intriguingly suggests that the unidentified mutation has compromised ATM function in germination as cell cycle activation and the DDR are both under control of ATM. Ideally, the analysis of a third *vtc2* mutant would clarify the conflicting phenotypes observed in this study.

8.5 Conclusions

Understanding the molecular basis of seed quality is an important strategy for enhancement of crop resilience and increased yields. Seeds provide the majority of global calorific intake and are required for propagation of most crop species. Determining the molecular changes which occur in seeds subject to different environment conditions during seed development, storage and germination will underpin biotechnological and plant breeding approaches to enhance seed performance.

The work presented in this thesis aimed to improve our understanding of the relationship between genome stability and the genetic basis of seed quality. The characterisation of molecular changes in the seed as a response to detrimental environmental conditions allows us to better predict how seed quality will change in response to rapidly changing temperatures. The work invested in this field will help improve crop performance and increase efficiency of seed conservation efforts in wild species. Increasing our knowledge of the interactions in the dry seed and upon imbibition could lead to the development of crop species that are resilient to perturbation, with enhanced germination and storability. Furthermore; by utilising methods, such as the ones undertaken within this thesis, robust biomarkers that predict seed quality can be developed. Due the high conservation of DNA repair and response mechanisms, and the central importance of DNA repair to control of germination and seed viability, further elucidation of these processes will provide the foundation for improved prediction and genetic enhancement of seed quality.

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