Roles and mechanisms of DNA repair factors and pathways in Maintaining Seed Quality

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds
Faculty of Biological Sciences
School of Biology

October, 2015

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgements

I would like to thank my supervisor Dr Christopher West for all his advice, support and incredible patience throughout my PhD. I would also like to express my gratitude to Dr Wanda Waterworth for all of her encouragement and guidance with my work. Thank-you to all of my colleagues at the University, in particular, Robbie Gillett, Thomas Lanyon-Hogg, Thomas Torode, Valerie Tennant, Vincent Agboh, Grace Hoystead, Jack Goode, Debs Roebuck. I would also like to thank Sue Marcus and all other members of the Knox, Foyer and Baker groups for their support.

I owe a great debt to my friends outside the University for their support, including Brown, Broadley and all my friends from home. A big thank-you must also go to Maria and Dave Duxbury (particularly for driving me to the viva) and all associates of The Crown Inn for listening to my ramblings and to all of my incredibly supportive friends.

Lastly, I would like to thank Kerry Rostron for her love and support through-out the tough times, my mother Julia, father Joe, sisters, Emma and Isobel, my Grandparents Mavis and Max, and all members of my family, all of whom I appreciate very much.

Abstract

Successful germination is a major determinant of crop yields and survival of plants in the natural environment. Our knowledge of molecular factors important to seed quality is far from complete, yet such understanding is vital in the endeavour of mitigating the detrimental effects of extended storage on seed vigour and viability. Genome integrity is crucial for cellular survival and transmission of genetic information. A number of pre-genomic era studies identified strong correlations between DNA damage accumulated in the quiescent seed and seed ageing. If unrepaired, DNA damage results in delayed growth, mutagenesis and cell death. Damage products incurred by DNA are remarkably heterologous and plants have evolved multiple pathways to facilitate the repair of specific damage products. Through the isolation and analysis of knockout *Arabidopsis* mutants, this work identifies and characterises DNA repair genes whose action is required during seed imbibition. These studies examined the importance of four major DNA repair pathways in germination and seed quality. Mutants deficient in non-homologues end joining (*KU70* and *KU80*), homologues recombination (*XRCC2*) and base excision repair (*ARP*) DNA repair pathways were all found to be hypersensitive to accelerated ageing treatment but those deficient in nucleotide excision repair (*ERCC1*) were not. Therefore this work establishes roles for multiple DNA repair pathways in seed longevity. Comparative analysis also defined non-homologues end joining as the most important DNA repair pathway to seed quality. *LIG6* encodes a unique plant specific DNA ligase with roles in seed longevity and implicated in repair of DSBs but remains largely uncharacterised to date. Here studies using extra-chromosomal recombination assays demonstrate *LIG6* functions in the promotion of recombination activities in *Arabidopsis* protoplasts. Further studies demonstrate the involvement of *LIG6* in the maintenance of root meristem genome stability. Collectively, this work provides an increased understanding of the early events central to the germination process and seed longevity.

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Abbreviations

5'-dRP	5'-deoxyribose-5-phosphate
6-4PP	pyrimidine (6-4) pyrimidones
8-oxoG	7,8-dihydro-8-oxoguanine
AA	accelerated ageing
ABA	abscisic acid
ABI	abscisic acid insensitive
ABRE	ABA response element
Alt-EJ	alternative end joining
ARP	apurinic endonuclease-redox protein
ASK1	*Arabidopsis* Skp1-like 1
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and rad4 related
B-NHEJ	backup non-homologous end joining
BER	base excision repair
BIR	break induced replication
bp	base pairs
BRCA2	breast cancer 2, early onset
CD	controlled deterioration
CDK	cyclin-dependent kinase
CHK1	checkpoint kinase 1
Col-0	Columbia 0 (an ecotype - the most widely-used wild type of *Arabidopsis thaliana*)
COM1	completion of meiotic recombination protein 1
CPD	cyclobutane pyrimidine dimers
CtIP	CtBP-interacting protein
dCK	deoxycytidine kinase
DDR	DNA damage response
DOG1	delay of germination 1
DMC1	DNA meiotic recombinase 1
DNA	deoxyribonucleic acid

DNA-PKcs	DNA-dependent protein kinase
DSB	double strand break
DSBR	double strand break repair
dsDNA	double stranded DNA
dNKs	deoxyribonucleoside kinases
dNTP	deoxyribose nucleotide triphosphates
dsRED	red fluorescent protein from *Discosoma* sp.
ECR	extra chromosomal recombination
EDTA	ethylenediaminetetraacetic acid
ERCC1	excision repair cross-complementation group 1
EMS	ethylmethane sulfonate
GFP	green fluorescent protein
GA	Gibberellic acids
GUS	β-glucuronidase
(d)HJ	(double) Holliday junction
H2AX	histone 2A isoform
HR	homologous recombination
HTA10	histone H2A 10
HUB1	histone monoubiquitination 1
IR	ionising radiation
JA	Jasmonic acid
KU	Ku antigen
LB media	Luria Bertani medium
LB	left border
LEC1	leafy cotyledon 1
LIG4	DNA ligase 4
MGT	mean germination time
MMC	mitomycin c
MMEJ	microhomology-mediated end joining
MMS	methyl methanesulfonate
MMR	mismatch repair
MRE11	meiotic recombination 11
MRN	MRE11/RAD50/NBS1 complex
MSH	MutS protein homolog

MSR	methionine sulfoxide reductase
MS	Murashige + Skoog basal medium
NADP	Nicotinamide adenine dinucleotide phosphate
NBS1	Nijmegen breakage syndrome
NER	nucleotide excision repair
NER-GGR	nucleotide excision repair – global genomic repair
NER-TCR	nucleotide excision repair – transcription coupled repair
NHEJ	non-homologous end joining
NMR	nuclear magnetic resonance
NO	nitrous oxide
PARP	poly-ADP-ribose polymerase
PCD	programmed cell death
PCNA1	proliferating cellular nuclear antigen 1
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium iodide
PIKK	phosphoinositide-3-kinase-related protein kinase
PIMT	protein L-isoaspartyl methyltransferase
POL	polymerase
PPP	pentose phosphate pathway
RAD	radiation sensitive
ROS	reactive oxygen species
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RNR	ribonucleotide reductase
SDSA	synthesis-dependent strand annealing
SAE2	sporulation in the absence of spo eleven
SOG1	suppressor of gamma response 1
SSA	single-strand annealing
SSB	single-strand break
ssDNA	single-strand DNA
SSP	seed storage protein
T-DNA	transfer DNA
TE	10 mM Tris-Cl 1mM EDTA pH 8.0

TFIIH	transcription factor – IIH
TK1	thymidine kinase 2
TSO2	TSO means ‘ugly’ in Chinese 2
UV	ultra violet
WT	wild-type
XP	xeroderma pigmentosum
XRCC	X-ray repair cross-complementing protein
XRI1	x-ray induced transcript 1
WAPL	wings apart-like
WEE1	WEE1 G2 Checkpoint Kinase

1. Introduction

1 Introduction

The success of seed germination and the establishment of a robust seedling are determining features for the propagation of plant species, of both ecological and economic importance. Germination is a critical stage in the plant life cycle due to the high susceptibility of the seedling to stresses including low temperature, high salt or acid soils, infection and physical damage. Rapid seedling establishment is of paramount importance as it underpins the propagation of high quality mature plants.

1.1 Seed Biology

A seed is an embryonic plant enclosed in a protective outer covering called the testa (seed coat). It is a characteristic of spermatophytes i.e. seed producing plants and the formation of the seed completes the process of reproduction in seed plants. This starts with the development of flowers and pollination and ends with the embryo developed from the zygote encased within a seed coat formed from the integuments of the ovule. Spermatophytes can be classified into two sub-groups which are known as angiosperm and gymnosperm (flowering plants and coniferous plants (and related clades) respectively).

1.1.1 Classification of seeds

Seeds are classified into three categories, orthodox, recalcitrant and intermediate, based on their storage behaviour and desiccation tolerance (Roberts, 1973; Ellis et al., 1990). Orthodox seeds withstand desiccation during maturation on the mother plant and retain their germination potential for extended periods of dry storage and chilling. Recalcitrant seeds cannot survive prolonged periods of drying or chilling and must remain hydrated during storage before germination. Intermediate seeds fall between the two previously described categories. Orthodox seeds are particularly important agromonically and consist mostly of annual and biennial crops such as the cereals and grain legumes, as well as agroforestry.

1.1.2 Orthodox seeds and *Arabidopsis*

From an evolutionary perspective, seed desiccation tolerance correlates with growth habitats in drier environments along with seasonal climates where seeds enable survival over the winter period. Orthodox seeds are commonly used in agriculture and this unique property of survival during dry storage allows the farmer flexibility over when seeds are sown. Preservation of embryonic viability in the dry state can extend over centuries in some species (Shen-Miller et al., 2002). This is associated with the existence of specific mechanisms to maintain the state of metabolic quiescence in mature dry seeds whilst preserving the integrity of cellular structures until cell metabolism is activated during germination.

Mature dry seeds of most species require a period of dry storage known as after-ripening to release them from dormancy, which evolved as a mechanism to prevent germination whist still on the mother plant (Iglesias-Fernández et al., 2011). Dormancy is a physiological state in which fully mature seeds that have taken up moisture and are metabolically active will not germinate under conditions of humidity, light, and temperature that are favourable to their germination. Seeds must first lose dormancy and meet these permissive conditions before germinating. Consequently it is thought seed dormancy evolved to postpone germination during unfavourable seasons (section 1.1.2.2).

This research primarily focuses on the orthodox Brassicaceae, *Arabidopsis thaliana* seed (hitherto referred to as *Arabidopsis*), [figure 1-1]. *Arabidopsis* is a small flowering plant (usually growing to 20–25 cm tall) native to Europe, Asia, and north-western Africa. It also appears to be native in tropical afroalpine ecosystems. It has a relatively small genome of approximately 135 megabase pairs (Mbp) and can complete its entire lifecycle in six weeks. The central stem that produces flowers grows after approximately three weeks, and the flowers naturally self-pollinate. Siliques are produced after fertilisation and are around 5–20 mm long, containing 20–30 seeds each. One *Arabidopsis* plant can produce several thousand seeds.

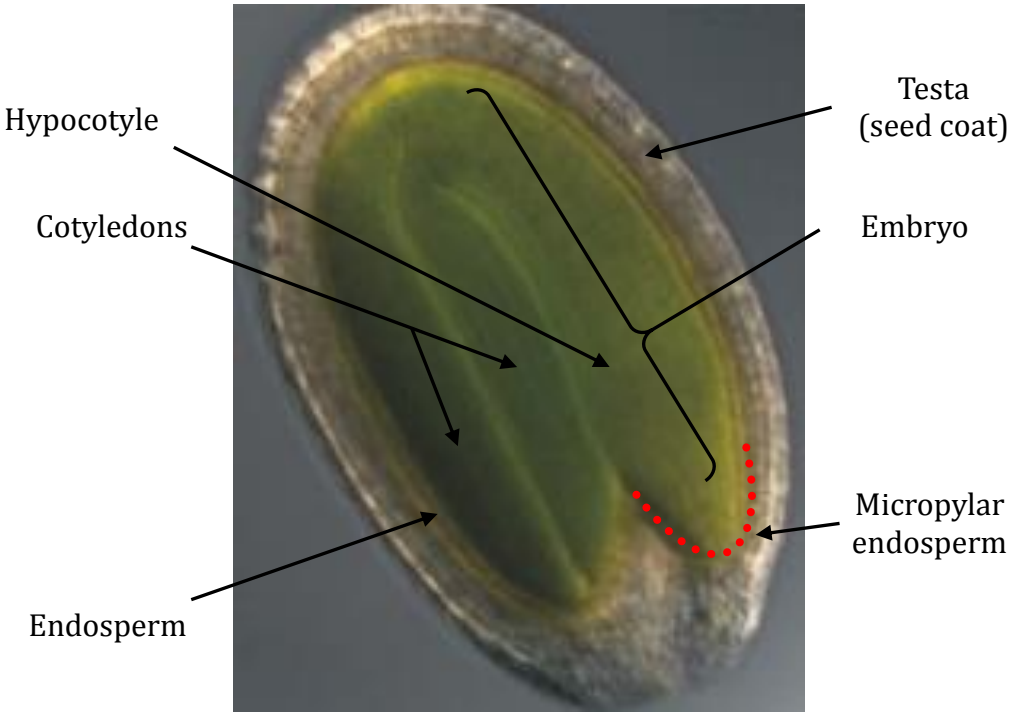


Figure 1-1 Structure of a physiologically mature *Arabidopsis* seed

A diagram highlighting key structural components of a mature *Arabidopsis* seed that has yet to undergo desiccation (section 1.1.2.1). Image adapted from (Junker et al., 2012). The testa, or seed coat, is the outer protective layer of the seed, developed from the integuments of the ovule, diploid maternal tissue. The embryo consists of diploid cells as a direct result of fertilization. The mature embryo consists of the cotyledons (seed leaves), hypocotyl (stem-like embryonic axis below the cotyledons) and the radicle (embryonic root). *Arabidopsis* is classified as a dicotyledon due to the presence of two embryonic leaves. In dicots such as *Arabidopsis,* the energy rich endosperm is immediately absorbed by the enlarged cotyledons and is the primary food source for the *Arabidopsis* embryo during germination. Endosperm cells are triploid (3n) with two thirds of an endosperm cell's genome being of maternal origin. The micropyle is a porous end in the endosperm and testa that serves to let water through during imbibition. During germination the *Arabidopsis* testa ruptures at the micropylar end and the radicle protrudes through the micropylar endosperm. The micropyle also plays an important role in fertilisation of the ovule as it is through here which the pollen tube usually passes.

The morphology of the *Arabidopsis* seed is presented in [Figure 1-1]. The endosperm is a tissue surrounding the seed embryo. The amount of endosperm in mature seeds is highly species-dependent and varies from an abundant endosperm layer, as is the case in *Nicotiana tabaccum* (tobacco), to a single layer, as shown in the diagram for *Arabidopsis thaliana*. In non-endospermic seeds such as *Pisum sativum* (pea), the cotyledons serve as sole food storage organs during germination. In angiosperms such as *Arabidopsis* the double

fertilization results in formation of the diploid embryo and the triploid endosperm. The embryo is enclosed by the testa, which provides a protective physical and mechanistic barrier between the embryo and outside the seed.

1.1.2.1 Seed maturation, desiccation, storage and potential damage

It is important to understand the molecular processes that are associated with the different stages of an orthodox seed's life cycle, as well as the mechanisms utilised to ensure protection against any sustained damage. The requirement for these protective mechanisms is exemplified during desiccation and imbibition. The extreme dehydration and rapid rehydration associated with maturation and imbibition respectively, are associated with high levels of oxidative damage to molecular structures and macromolecules (Bailly, 2004; Kranner et al., 2010a; Waterworth et al. 2011). Furthermore during dry storage, seeds remain quiescent with low metabolic activity. Low levels of molecular damage can accumulate during this period, and over time this can lead to a delay in germination (lower vigour) and a decrease in seed viability (Kranner et al., 2010a; Waterworth et al., 2015).

Further to the oxidative damage that can occur during seed desiccation, low moisture content can cause cellular damage due to protein unfolding and membrane disturbance. Sugars, late embryogenesis abundant proteins, and heat shock proteins play important roles in preventing this damage in seeds (Hoekstra et al., 2001).

During the maturation phase, seeds accumulate storage reserves and become desiccation-tolerant and dormant (section 1.1.2.2) whilst they develop on the mother plant, which is initiated after the embryo has fully developed (Finch-Savage and Leubner-Metzger, 2006). At least four central regulators are known to tightly control seed maturation in *Arabidopsis*: *ABSCISIC ACID-INSENSITIVE 3* (*ABI3*), *LEAFY COTYLEDON 1* (*LEC1*), *LEAFY COTYLEDON 2* (*LEC2*), and *FUSCA 3* (*FUS3*) (Santos-Mendoza et al., 2008). When any one of these four genes are knocked out, common phenotypes are observed including reduced expression of seed storage proteins (Gutierrez et al., 2007) and decreased dormancy at

maturation (Raz et al., 2001). Other factors have been identified that have more specific roles in one of the maturation processes including two genes required for seed dormancy establishment: *DELAY OF GERMINATION 1* (*DOG1*) and *HISTONE MONOUBIQUITINATION 1* (*HUB1*) (Bentsink et al., 2006; Liu et al., 2007) .

During *Arabidopsis* seed development different programs of gene expression have been identified, comprising distinct classes that are co-ordinately regulated. The maturation class, expressed at early and mid-maturation phases, includes major seed storage protein (SSP) genes (including 2S albumins and 12S globulins). The late embryogenesis abundant (LEA) class, which includes primarily genes involved in the acquisition of desiccation tolerance, is expressed during the later stages of seed maturation (Holdsworth et al., 2008)

Abscisic acid (ABA) is an important positive regulator of both the induction of dormancy during seed maturation and the maintenance of the dormant state in imbibed seeds following shedding (Finch-Savage and Leubner-Metzger, 2006). Deficiency of ABA during seed development is associated with the absence of primary dormancy in the mature seed, whereas the over-expression of ABA biosynthesis genes can increase seed ABA content and enhance seed dormancy or delay germination (Nambara and Marion-Poll, 2003). Gibberellins (Gibberellic acids, GA) are required for plant embryogenesis however in later phases of embryogenesis their synthesis must be down-regulated as dormancy is initiated. Cross-talk with other plant hormone pathways, such as ABA, ethylene or auxin are involved in this regulation of GA biosynthesis, but as yet these interactions are not fully understood (Holdsworth et al., 2008).

The level of dormancy increases during maturation and reaches a maximum in harvest-ripe seeds. During subsequent dry storage of the seeds, dormancy status reduces until seeds are able to complete germination when imbibed under favourable conditions; this process is known as after-ripening. The speed of after-ripening can vary depending on environmental conditions during seed maturation, seed storage and germination (Holdsworth et al., 2008). The molecular mechanisms that control after-ripening are not well understood,

however several recent studies have provided evidence of regulation at various levels including RNA transcription. Transcripts of all ontological categories were observed; however, the 2–3% (500 genes) most highly expressed genes encode functions mainly associated with metabolism, protein synthesis and degradation. The ABA response element (ABRE) was over-represented in the promoters of genes highly expressed in the dry seed transcriptome (Nakabayashi et al., 2005). Whether or not seeds are capable of transcribing DNA and synthesising proteins in the dry state, for example, during prolonged after-ripening, remains to be demonstrated unequivocally. However work in sunflower has demonstrated levels of a subset of transcripts can differentiate between dormant and after ripened seeds (Meimoun et al., 2014). Transient, low-level transcription and translation may occur in air-dry, low-hydrated seeds, with more than 12,000 mRNA species being present in dry seeds of *Arabidopsis.*

1.1.2.2 Seed dormancy and hormonal control of germination

Plant hormones have controlling roles in the regulation of seed germination and dormancy. In turn, the genes encoding proteins that modulate the metabolism of these signalling molecules, in particular those involved in the biosynthesis and deactivation of GA and ABA, are major genetic regulators of these events.

Removal or deactivation of ABA is also important as interactions between this hormone and GA play a regulatory role, inhibiting germination progression. A restraint on the completion of germination in seeds of some species is imposed by the surrounding structures, e.g. the endosperm, and thus there is a requirement either for it to be enzymatically weakened to allow the radicle to emerge, or for sufficient force to be generated within the embryo axis to physically break through, or both (Nonogaki et al., 2010). The endogenous ABA contents of non-dormant and dormant seeds rapidly decline upon imbibition during the early phase of germination (Preston et al., 2009). However, in dormant seeds this decrease is stopped and *de novo* ABA synthesis in the

imbibed state causes higher ABA contents, which are required for dormancy maintenance and for inhibiting germination (Nambara et al., 2010). Decreased levels of ABA in a seed during imbibition and early phase II is a major prerequisite for the completion of germination. ABA treatment of *Arabidopsis* seeds which have been after ripened showed that ABA does not affect the kinetics of testa rupture, but rather inhibits endosperm weakening and rupture (Müller et al., 2006). Changes in hormone contents during the early germination phase are also evident in *Arabidopsis* seeds for jasmonic acid, where content decreases, and indole-3-acetic acid (IAA) where content increases (Preston et al., 2009).

The germination-inhibiting effect of ABA is counteracted by gibberellins (GAs) and by ethylene (Holdsworth et al., 2008). Ethylene has important roles during the late phase of germination and counteracts the ABA inhibition by interfering with ABA signalling, doing so without affecting ABA content (Linkies et al., 2010). In contrast, GAs are important during the early and the late phase of germination and counteracts ABA inhibition. GA biosynthesis localises to the radicle, hypocotyl, and micropylar endosperm during germination. Due to the rapid ABA degradation, the GA/ABA ratio increases approximately three-fold during early germination and around ten-fold during late germination (Ogawa et al., 2003).

1.1.3 Physical, morphological and biochemical changes associated with germination and radicle emergence

The success of seed germination and the establishment of normal seedlings are determining features for the propagation of a plant species as well as crop yields. Germination and the period shortly after (termed seedling establishment), is the most critical stage of a plant's lifecycle due to the vulnerability of germinating seedlings. This includes mechanical injury, disease and environmental stresses. Rapid, uniform germination correlates with strong healthy seedlings which subsequently rapidly establish themselves (Rajjou et al., 2012). Germination performance consists of two qualities; viability (the maximum germination capacity of a seed lot) and vigour (the rate and

uniformity at which a seed lot will germinate). By definition, the process of germination in orthodox seeds encompasses events in the embryo starts with imbibition, the rapid uptake of water by a dry quiescent seed and ends with radicle protrusion through the seed coat (Bewley and Black, 1994). Seed lots with good germination performance are of great importance in the agronomical world. In seeds under conditions of stress such as high salinity; delayed onset, reduced rate, and increased dispersion of germination events results. This leads to reductions in plant growth and final crop yield, something exacerbated in seed lots with poor germination performance (Powell and Matthews, 2012; Rajjou et al., 2012; Waterworth et al., 2015).

Germination is a complex process during which the seed must quickly recover physically from maturation drying, resume a sustained intensity of metabolism, complete essential cellular events to allow for the embryo to emerge and prepare for subsequent seedling growth. The transition from a dry seed to a seedling is tri-phasic according to the uptake of water [Figure 1-2].

Early on following the start of Phase I (imbibition), there is re-establishment of metabolism; restitution of the chemical and structural integrity of cells requires the co-participation of synthetic and protective events (Rajjou et al., 2012). Protein synthesis and respiratory activity initially involves components stored within the mature dry seed, although transcription and translation commence early during imbibition, as shown by transcriptome and metabolome analyses (Gallardo et al., 2001).

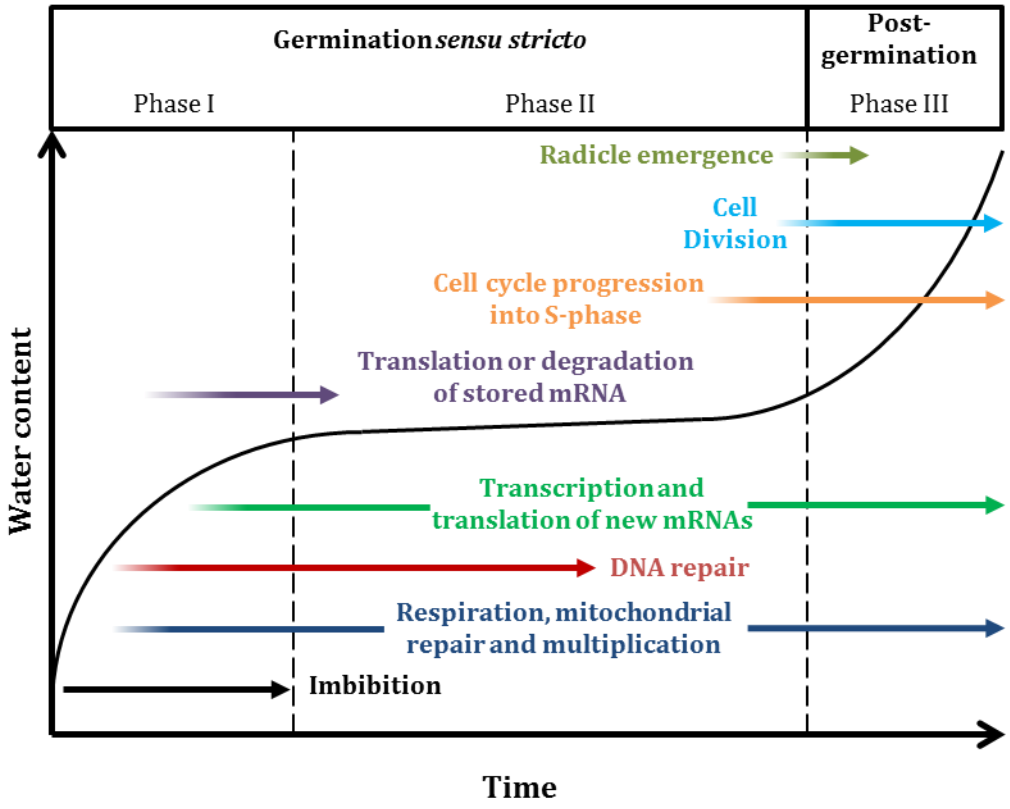


Figure 1-2 Phase division of Germination

The division of germination into the three stages as classically defined (Bewley and Black, 1994). Time course of physical and metabolic events that occur during germination (Phases I and II) and early seedling growth (Phase III). The time taken for these events to occur varies between species and is influenced by germination conditions. The curve shows a stylized time course of water uptake. Adapted from (Nonogaki et al., 2010).

1.1.3.1 Phase I: germination and resumption of metabolic activities

Rehydration during phase I of germination involves the hydration of matrices including the cell walls and reserve polymers [Figure 1-2]. Before imbibition an *Arabidopsis* dry seed is approximately spherical with a diameter of 200 μm and then swells to an ellipsoid with an approximate length of 350 μm and diameter approximately 250 μm (Meinke, 1994; Robert et al., 2008). Work in imbibed *Nicotiana tabacum* (Tobacco) seeds using proton nuclear magnetic resonance (NMR) micro-imaging has shown that an increased water content with time does not a result from a uniform increase in the hydration of all cells, but rather an increase in the number of cells which become hydrated within the seed. Water first reaches the micropylar endosperm and radicle tip and it is thought this leads to a rapid resumption of metabolic activities in these regions (Manz et al., 2005). The pattern of water uptake through the micropyle is mirrored in other seeds including wheat (Rathjen et al., 2009) and pea (Wojtyla et al., 2006).

Whilst water uptake is rapid in orthodox seeds, there is a more gradual resumption of steady state metabolism. That being so, large changes in metabolic activity are observed from the early onset of imbibition. Metabolism is reactivated with enzymes that were stored in the seed during maturation. In *Arabidopsis* proteomic analysis has shown a large number of enzymes involved in the major metabolic pathways were found in dry seeds and remained stable or even accumulated further during early germination (Gallardo et al., 2001; Fu et al., 2005; Rajjou et al., 2008). Proteomic analysis in other plant species has demonstrated the presence of proteins important for metabolic activity in dry seeds, including *Lepidium sativum* (cress) (Muller et al., 2010), *Beta vulgaris* (sugar beet) (Catusse et al., 2008), *Hordeum vulgare* (barley) (Sreenivasulu et al., 2010) and (*Oryza sativa*) rice (Yang et al., 2007).

Resumption of energy metabolism occurs early during phase I of germination. Dry mature lettuce seeds contain mitochondria that have poorly differentiated inner-membranes. However, active enzymes are present and likely to provide

sufficient ATP for several hours after the start of imbibition (Hourmant and Pradet, 1981). In the dry seed, these enzymes are protected by LEA proteins found in the mitochondria (Tolleter et al., 2007).

Imbibition is also associated with temporary anaerobic conditions in the plant embryo as the seed coat is impermeable to oxygen during the early hours of germination (Kennedy et al., 1992). The pentose phosphate pathway (PPP) and glycolysis respiratory pathways are also activated during seed imbibition (Nonogaki et al., 2010). The glycolytic pathway is predominant when there is low oxygen availability, and so mitochondrial ATP production is restricted. In contrast, when mitochondria become active, the PPP pathway predominates (Roberts, 1964). Interestingly, a key link between the two pathways is the oxidised pyrimidine nucleotide NADP, a coenzyme of glucose-6-phosphate dehydrogenase. The metabolic flux between the two pathways may be accelerated by nitrogen-containing compounds that are important in the release of dormancy such as NO (section 1.1.2.2), by indirectly increasing NADPH oxidation (Nonogaki et al., 2010), providing a potential link between the seed's decision to progress through germination and metabolic activity.

Upon imbibition, dramatic changes in the transcriptome can be observed after as little as 1 to 3 hours in *Arabidopsis* (Preston et al., 2009). Some changes have been shown to be tissue specific, demonstrating strong regulation of the transcriptome very early in germination (Okamoto et al., 2010). Common *cis*-acting elements in gene promoters have been found for transcripts that show strong changes in abundance, displaying similar expression patterns during these early germination events (Okamoto et al., 2010). In rice, transcripts that display strong regulation between zero and three hours imbibition are enriched for 3' untranslated regions (UTRs) with motifs associated with RNA stability.

1.1.3.2 Phase II: pre-germination and radicle emergence

Once the rate of water uptake and changes in seed size and shape start to plateau, germinating seeds move into water uptake phase II, during which the water content remains stable and can vary widely in duration. Phase II ends

with testa rupture and germination. This is followed by phase III water uptake following radicle protrusion and completion of germination *sensu stricto*. Phase III water uptake continues during the transition to seedling growth [Figure 1-2].

The endosperm in mature seeds provides an additional tissue that regulates germination by ABA in response to environmental factors. A two-step germination process appears to be a phylogenetically widespread trait determined by the anatomy of the seed-covering layers (Linkies et al., 2010). While *Arabidopsis* seed coat development has been studied in detail (Haughn and Chaudhury, 2005), little is known about the changes of the seed coat's mechanical and biochemical properties that ultimately lead to testa rupture, [Figure 1-1] initiated at the micropylar seed end.

Embryo cells elongate prior to the completion of seed germination in *Arabidopsis* and cell division is not evident in the embryo during germination (Barrôco et al., 2005). After the initial swelling is complete, all changes in seed size and shape during germination are caused by cell expansion. Expanding plant cells adjust the extensibility of their cell walls by remodelling the major components of the cell wall. Loosening of the wall allows water influx which drives cell expansion and generates cellular turgor pressure (Schopfer, 2006). It is proposed that elongation of the *Arabidopsis* embryo occurs in a distinct elongation zone between the radicle and the lower hypocotyl [figure 1-1] (Schopfer, 2006).

1.1.3.3 Germination and cell cycle

As previously stated cell division in the *Arabidopsis* embryo is not required for germination and does not occur until radicle emergence. However cell cycle regulation is a requirement. Mitotic divisions are observed only after the radicle has protruded and presumably rely on the *de novo* production of other cell cycle regulators. Flow cytometry of germinating *Arabidopsis* seeds indicated that DNA replication is mainly initiated at the onset of root protrusion (Barrôco et al., 2005), whilst analysis has shown synthesis and/or activation of a subset of core cell cycle proteins trigger DNA replication, but is not sufficient

to drive cells into mitosis (Masubelele et al., 2005). Cell cycle control during seed germination may be exerted at multiple levels. However, knowledge of cell cycle events and their importance for germination is still scarce and fragmentary. Further to this, different species may have developed unique control mechanisms which are more suited to specific germination characteristics and habitat (Vázquez-Ramos and de la Paz Sánchez, 2003).

1.1.4 Seed quality, vigour and longevity

Seed quality is a measure of a seed lots storability (longevity), ability to germinate rapidly and uniformly (vigour) and to do so with high viability (Rajjou et al., 2012). It has only been in recent years we have begun to identify molecular determinants of seed quality (Barrett et al., 2015).

Although proteins and membranes are protected in the mature dry seed, damage still occurs during maturation drying and imbibition. Cellular membrane structures that are maintained in a gel phase during maturation drying undergo a transition to a liquid crystalline phase when rehydrated. Damage to membranes can however occur during the transition between the two phases and this results in the leakage of solutes from cells (Nonogaki et al., 2010). To this point, initial imbibition is often accompanied by a substantial leakage of cellular solutes. Similar phenomena can be observed in resurrection plants and pollen that rapidly return from a dry quiescent state to a fully hydrated state (Hoekstra et al., 1999). This leakage can actually help accelerate germination by lowering inhibitor concentrations within a seed (Matilla et al., 2005). Too much leakage can have disadvantageous effects and may be considered a sign of damage to membranes and cellular compartments caused by rapid and uncontrolled rehydration (Matthews and Khajeh-Hosseini, 2007)

In order to deal with the damage imposed during dehydration, storage and rehydration, seeds activate a number of repair mechanisms during imbibition. Damage to proteins can occur during desiccation and/or ageing during seed storage (section 1.1.4.1) due to the formation of modified amino acids. This may cause protein misfolding or a reduction or even loss in protein function. Oxidation within the seeds has important roles from maturation to

germination, however, detrimental effects associated with seed ageing are observed if oxidative levels in the seed become too high (Clerkx et al., 2004). A common type of oxidative damage occurs on methionine residues forming methionine sulfoxide. This damage is reversible through the actions of a class of enzymes called methionine sulfoxide reductases (MSRs). Transgenic *Arabidopsis* seeds with altered *MSR* expression displayed a strong positive correlation between MSR capacity and seed longevity (Châtelain et al., 2013).

Other protein repair mechanisms in *Arabidopsis* have been identified as potentially conferring longevity in seeds. The protein L-ISOASPARTYL METHYLTRANSFERASE (PIMT) catalyses the conversion of abnormal L-isoaspartyl residues to the physiologically normal L-aspartyl form (Dinkins et al., 2008). *Arabidopsis* seeds overexpressing *PIMT1* (one such gene encoding this class of enzyme) caused a reduction in the accumulation of abnormal residues in proteins, and furthermore resulted in seeds with both increased vigour and longevity. Conversely a reduction in expression of this gene increases the accumulation of L-isoaspartyl residues in proteins within freshly harvested dry mature seeds. This accumulation leads to reduced seed vigour under germination stress conditions and hypersensitivity to ageing treatments (Oge et al., 2008).

Levels of DNA damage during seed desiccation and storage correlate with seed quality (Cheah and Osborne, 1978b). Damage and repair to genomic DNA is covered more extensively in later sections but it includes the progressive loss of telomeric sequences during prolonged dry storage (Boubriak et al., 2007) along with the requirement for DNA repair machinery to repair double strand breaks. A failure to do so leads to loss of seed viability and vigour (Waterworth et al., 2010).

Seed vigour and longevity have not been intensively targeted in breeding programs and understanding of the molecular basis of seed quality is far from complete. Identification of more genes associated with seed quality is required in order to ensure that these traits can be improved through molecular breeding. Current understanding has elucidated the need for the repair of

accumulated damage in orthodox seeds before germination can run to completion, this is known as the repair hypothesis (Matthews et al., 2012).

1.1.4.1 Mean germination time correlates with seed lot quality

Germination is considered to have completed at the point of testa rupture and radicle emergence (Roberts, 1972a). In germination, the lag period is defined as the observed time from the start of imbibition to radicle emergence. In maize, the lag period is proportional to the extent of deterioration in a seed lot determined by overall viability and seedling growth (Matthews and Khajeh-Hosseini, 2007). Germination curves showing seed vigour are produced following regular counts during germination of a seed lot from the start of imbibition to radicle emergence. These curves have demonstrated that seed vigour can differ between seed lots, even between those that display rapid, vigorous germination (Matthews and Powell, 2012). The speed of germination can also be expressed as a mean germination time (MGT) value (Ellis and Roberts, 1980), which is a measure of the average time it takes for a seed to germinate.

MGT is an important measure in the agronomical world in the evaluation of seed quality in numerous crops species. Work in *Zea mays* (maize), *Capsicum annuum* (pepper), *Citrullus lanatus* (watermelon), *Cucumis sativus* (cucumber) and *Brassica napus* (oil seed rape) have all demonstrated MGT is highly indicative of seedling field emergence rate as well as seedling establishment, size and uniformity (Matthews et al., 2012). Seed vigour and seedling establishment have long been known to be key determinants of crop yield (Ellis and Roberts, 1980).

1.1.4.2 Seed ageing and the repair hypothesis

Seed longevity determines seed vigour after storage. Initial work in the 1970's demonstrated, through protein synthesis assays using rye embryo cell extracts, that extended periods of storage decreases the ability of embryos to synthesise proteins *in vivo* and correlates with poor germination and eventual loss of

viability (Roberts and Osborne, 1973). In rye seeds a loss in viability was also associated with greater levels of DNA single strand breaks (SSBs) after storage (Cheah and Osborne, 1978a). This work has been built on by the International Seed Testing Association (ISTA) in developing the seed ageing/repair hypothesis to explain differences in vigour and viability (Matthews et al., 2012). In understanding seed quality and ageing, tests have been developed that closely mimic the effects of natural ageing but over a much shorter time period (Rajjou et al., 2008). Two commonly used examples are controlled deterioration (CD) and accelerated ageing (AA). These techniques rely on exposing the seeds to unfavourable storage conditions including high temperature and high relative humidity, which holds the seeds at a high moisture content (Powell and Matthews, 2012). These conditions induce damage to cellular macromolecules (e.g. DNA and proteins) and organelles within the seeds (Roberts. 1972; Roberts. 1973; Roberts et al. 1973; Dourado et al. 1984). Both of these tests exacerbate differences in vigour between seed lots and are commercially used as markers of seed longevity (Powell and Matthews, 2012).

As described in previous sections, high levels of molecular damage are accumulated during the different seed stages. The repair hypothesis states that the more damage a seed has to repair, the longer the lag period will be to allow the seed time to repair accumulated damage before germination is completed. Work in rye first showed that upon imbibition, DNA repair synthesis occurs. With seed ageing, the delay to radicle emergence is increased accompanied by an extended period of DNA synthesis (Osborne et al., 1984; Elder and Osborne, 1993).

1.1.4.3 Improving seed vigour

Work in many crop species has shown that after a period of hydration followed by drying back decreases the lag period (Rajjou et al., 2012). This treatment is known as seed priming, a technique that is widely used in the agronomic industry to improve the efficiency of seed germination and field emergence under adverse conditions (Rajjou et al., 2012). Osmopriming is performed by

soaking seeds in a solution of low water potential. This facilitates the controlled uptake of water allowing the initiation of metabolic activities in the seed embryo, whilst prohibiting radicle emergence and therefore completion of germination (Gallardo et al., 2001). In most orthodox seed-producing plant species, desiccation tolerance is maintained up until the point of radicle emergence. Therefore, priming can be followed by a dehydration step allowing extended storage of the primed seed over-coming prior deterioration (Butler et al., 2009). Only once seeds are exposed to solutions of higher water potential will germination proceed to completion. The long term benefits of priming treatments are still however widely debated. Work in rice (*Oryza sativa*) seeds has shown that the effects of priming are permanently offset by prolonged storage or unfavourable storage conditions (Hussain et al., 2015).

The osmotic agent polyethylene glycol (PEG) is commonly is used as a seed priming agent and in *Arabidopsis* this induces pre-germination metabolism (Gallardo et al., 2001). Further to this, work in sugar beet has shown seeds that have first undergone controlled deterioration (a process that artificially ages seeds causing loss of vigour and even viability – section 1.1.4.2) before priming, display reversible changes in protein accumulation patterns (Catusse et al., 2011). These proteins were shown to correlate with seed vigour, including proteins involved in several metabolic pathways for lipid and starch mobilisation, translation initiation factors involved in protein synthesis and components of ABA signalling pathways important in the cellular regulation of germination (section 1.1.3) (Catusse et al., 2011; Rajjou et al., 2012). Priming has also been implicated in initiating DNA repair in *Allium porrum* (leek) seeds. Using [^{3}H]-thymidine incorporation as a measure DNA synthesis, it was demonstrated that osmopriming causes DNA repair-type synthesis (Bray et al., 1989). This suggests that repair of DNA damage during germination is an important process.

1.2 DNA structure and the *Arabidopsis* genome

DNA is a polymer composed of repeated deoxyribonucleotide monomer subunits. These consist of a deoxyribose sugar molecule covalently linked to a phosphate residue at the 5' carbon and covalently bound to a nitrogenous base at the 1' carbon. When polymerised, the phosphate residue of each deoxyribonucleotide is also bound to the 3' carbon on the next deoxyribonucleotide in the polymer, which forms a backbone structure of sugar residues linked by phosphodiester bonds. DNA is made up of four nitrogenous bases: thymine and cytosine (pyrimidine bases) and guanine and adenine (purine bases). Genomic DNA is found in a double stranded, right-handed helical structure. The sugar-phosphate backbone of each strand runs in an anti-parallel direction to the other, with bases on the opposing strand pairing with each other in a complementary fashion: thymine (T) pairing with adenine (A) and cytosine (C) pairing with guanine (G) (Figure 1-3).

In an organism's genome, DNA encodes the genetic information for the synthesis of proteins in triplet code i.e. three bases encodes one amino acid (the building blocks of proteins). In 2000, *Arabidopsis* was the first multi-cellular eukaryote to have its genome completely sequenced. The genome was shown to comprise 1.25×10^8 base pairs, arranged into 5 chromosomes (*Arabidopsis* Genome, 2000).

Over 27 000 protein-coding genes are predicted in *Arabidopsis* (Swarbreck et al., 2008). In addition to these genes, a range of structural RNA species also are encoded by the *Arabidopsis* genome including rRNA and tRNA, as well as 889 pseudogenes and transposable elements (Swarbreck et al., 2008). DNA also provides the binding sites for components of transcription, therefore making it the basis for the control of gene expression. Over the past few decades, these non-protein encoding regions have been shown to play essential roles in plant development, physiology and other processes including the discovery of micro RNAs (miRNAs) which perform targeted regulation of specific mRNA expression required for plant development (Baulcombe, 2004) and response to environment stress (Zhang et al., 2005). The control of gene expression is also

regulated by the accessibility of the DNA, mediated by covalent modifications to the DNA bases and associated proteins known as epigenetic regulation. Epigenetic changes to DNA can be incredibly stable and indeed heritable, but in other circumstances they can also be tissue specific and transient in response to changing cellular conditions or stress (Grant-Downton and Dickinson, 2005; Meyer, 2011).

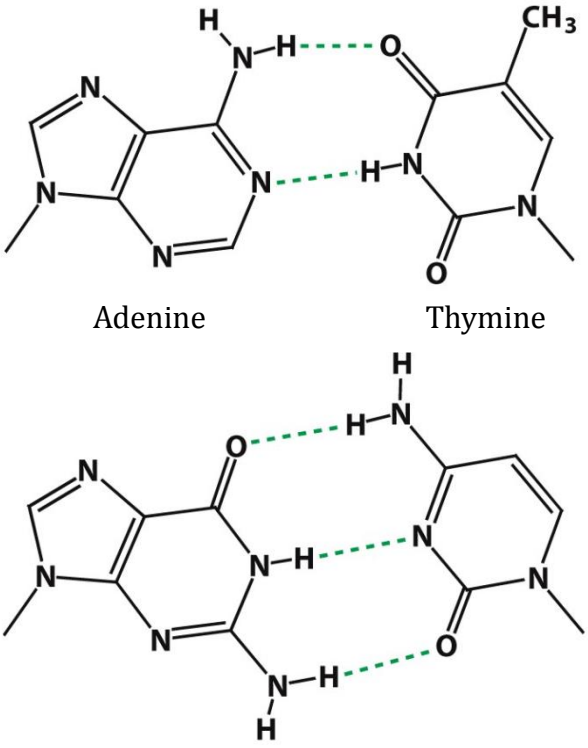


Figure 1-3 Complementary base pairing

Base pairs, which form between specific nucleobases, are the building blocks of the DNA double helix and contribute to the folded structure of both DNA and RNA. Dictated by specific hydrogen bonding patterns, Watson-Crick base pairs (guanine-cytosine and adenine-thymine) allow the DNA helix to maintain a regular helical structure that is subtly dependent on its nucleotide sequence.

1.3 Sources and types of DNA damage to plants

To ensure the integrity of genetic material and DNA structure, it is essential that plants, and indeed any organism, has a means of repairing any damage accumulated to the genome. This safeguards the genetic information so that daughter cells inherit an intact copy of each chromosome following DNA replication and cell division. Precise and accurate DNA replication is of particular importance in the plant vegetative tissues as these can give rise to the germline (Walbot, 1985). Plant growth is controlled in the root and shoot tips, these areas are known as meristems. The meristem contains an actively dividing group of undifferentiated cells, which gives rise to plant organs such as leaves and flowers. The plant germline is derived from these cells late in a plant's development. This means that mutations acquired during the life of vegetative cells can be passed to the next generation of plants (Ries et al., 2000).

Plant cells are subjected to particularly high levels of DNA damage that causes changes to the chemical structure of the DNA. These result from a sedentary lifestyle and a dependence on sunlight for energy along with the concomitant exposure to environmental stresses including UV-A, UV-B, ozone, desiccation, rehydration, air and soil pollutants including heavy metals. These genotoxic agents cause a range of DNA damage products including single-strand DNA breaks (SSBs) and DSBs. DSBs also occur spontaneously, arising from defects occurring during DNA replication including collapsed replication forks, replication past a SSB and steric stresses as DNA is unwound (Figure 1-4).

1.3.1 UV damage

UV (ultraviolet) light is a major cause of DNA damage in plants due to their sessile phototrophic nature, in which they are exposed to UV on a continual basis during daylight hours. The UV spectrum is the part of the electromagnetic spectrum between 10 – 400 nm. It is the UV-A (315-400 nm) and UV-B (280-315 nm) wavelengths that are able to penetrate the Earth's atmosphere from the Sun at levels detrimental to DNA integrity. UV induces dimerisation

between adjacent pyrimidine bases including pyrimidine-pyrimidone (6-4) and cyclobutane pyrimidine dimers photoproducts. These changes to DNA structure are cytotoxic as they block transcription and DNA replication (Chen et al., 1996).

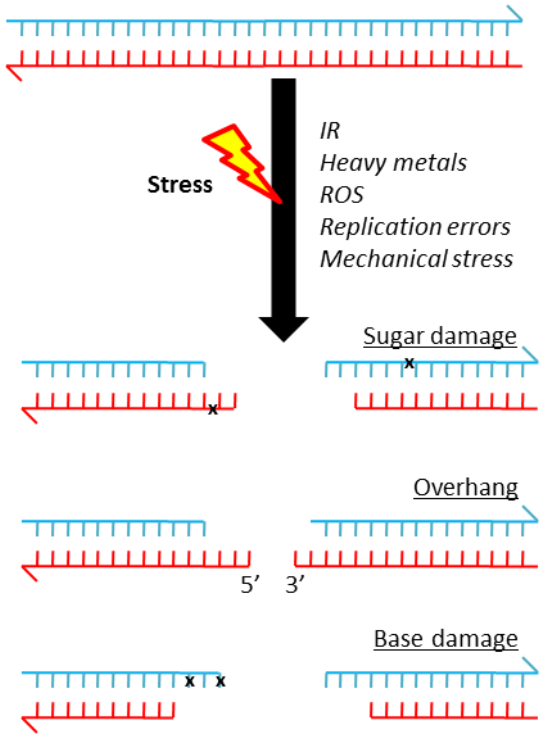


Figure 1-4 Sources of DNA damage and DSB products

A multitude of stresses can cause DSBs including ionising radiation (IR), heavy metals, reactive oxygen species (ROS), replication errors and mechanical stresses. This results in a wide range of DBS products including overhangs and damage to base and sugar residues. The ——→ denotes a 5' to 3' direction of DNA polarity.

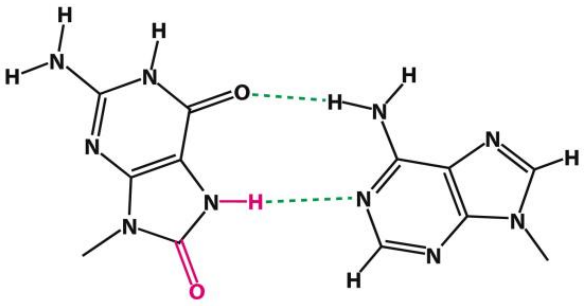
1.3.2 Alkylation and hydrolysis

DNA damage can also occur due to alkylation. An example of DNA alkylation is guanine methylation producing O^6-methylguanine. This change in itself is mutagenic as O^6-methylguanine is mis-paired with thymine rather than cytosine by the replication machinery (Chen et al., 1996). Exposing plants to alkylating agents including ethylmethane sulfonate (EMS) causes alkylation of nitrogenous bases and is routinely used to induce point mutations in the *Arabidopsis* genome. Using forward genetics, this technique allows identification of new players in a specific biological process or signalling pathway (Qu and Qin, 2014).

1.3.3 Reactive oxygen species and oxidative damage

In plants, reactive oxygen species (ROS) are constantly generated as a by-product of metabolic reactions that occur in the mitochondria, chloroplasts and peroxisomes (Foyer and Noctor, 2003). In the chloroplast, ROS are generated as a by-products of photosynthesis and include the superoxide radical ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) (Asada, 2006). Conversely in the dark, most of the ROS generated occurs in the mitochondria by the over-reduction of the electron transport chain in the form of $O_2^{\bullet-}$ (del Rio et al., 2006). In the peroxisome, the main reactive oxygen species generated are hydrogen peroxide (H_2O_2) (Roldán-Arjona and Ariza, 2009) and $O_2^{\bullet-}$ (Maxwell et al., 1999). ROS are also generated by peroxidases and oxidases in the cell and perform important roles in developmental regulation as well as in response to environmental challenges such as recognition of pathogens (Gapper and Dolan, 2006; Moller et al., 2007). ROS also play key roles in seed dormancy and germination, however, they can react with and have detrimental effects on cellular macromolecules including DNA.

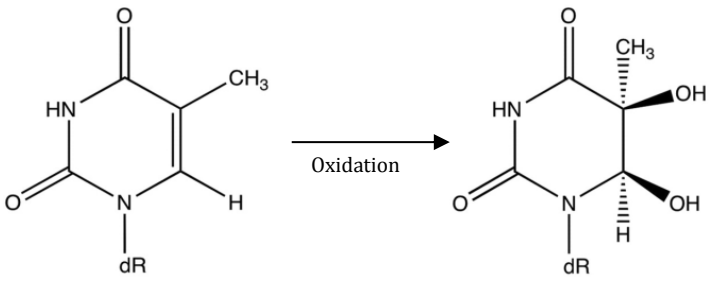
[A]



7,8-dihydro-8-oxoguanine

Adenine

[B]



Thymine

Thymine glycol

Figure 1-5 Oxidation of nucleotide bases

[A] 7,8-dihydro-8-oxoguanine-adenine base pairing. The presence of an 8-oxoG base in genomic DNA can lead to a G to T transversion mutation via an 8-oxoG:A base pair stabilised by two interbase hydrogen bonds.

[B] Thymine oxidation to Thymine glycol, Thymine glycol is a product of thymine oxidation. $^{\bullet}OH$ radicals attack the double bond of thymine at C-5 or C-6 and can abstract hydrogen methyl group (Simic, 1994). The hydroxythymine radical intermediate can react with O_2 to yield thymine glycol (Demple and Linn, 1982).

Oxidative damage by ROS to nucleic acids is caused by covalent modifications to bases and the sugar phosphate backbone, leading to changes in DNA chemical structure. ROS can attack deoxyribose units and cause DNA fragmentation. ROS can also cause single strand breaks mediated by hydroxyl radicals via the removal of hydrogen from the C_3, C_4 and C_5 of the 2-deoxyribose moiety in DNA (Moller et al., 2007). Hydroxyl radicals are highly reactive, not only to the 2-deoxyribose moiety but also the nucleobase of DNA. Subsequently, all four bases can be attacked by ROS and a wide range of oxidised base products have been discovered. The most common single oxidised base observed in living cells is 7,8-dihydro-8-oxoguanine (8-oxoG). This is a ubiquitous product of DNA oxidation generated by the reaction of hydroxyl radical and closely related pyrimidine peroxyl radical (Cadet et al., 2012). 8-oxoG itself is mutagenic as it mis-pairs with adenine leading to a base transition of G → T [Figure 1-5 A] (Chen et al., 1996). In addition to the mutagenic nature of 8-oxoG, thymine glycol oxidation, another type of ROS mediated DNA damage, is cytotoxic to a plant cell as it blocks replication [Figure 1-5 B] (Chen et al., 1996).

High energy irradiation (X-rays and γ-rays) can cause damage directly to DNA but also indirectly through generation of ROS, particularly through a radiolysis reaction with water ($H_2O \rightarrow OH^{\bullet} + H^{\bullet}$). These types of ionising radiation are produced by the decay of natural radioactive isotopes and cosmic rays that penetrate the atmosphere from space. The Chernobyl nuclear disaster has caused much higher levels of IR in contaminated areas and, interestingly, plants from these areas have shown adaptation in response to double strand breaks (DSBs) and an increased resistance to DNA damaging agents (Kovalchuk et al., 2004). In addition to ROS production through UV irradiation, ROS are also generated through the action of heavy metals in the Fenton reaction (Kovalchuk et al., 2001).

The production of ROS during desiccation and lengthy periods of storage is a major contributor to DNA damage and is associated with seed ageing (Balestrazzi et al., 2011b). To date most contributions in this field have been

concerned with the role of ROS in the loss of vigour and viability during the prolonged storage of desiccation tolerant orthodox seeds. Lipid peroxidation induced by these compounds has been widely cited as being a major cause of seed ageing (Priestley, 1986; Bailly et al., 2004). Desiccation-intolerant recalcitrant seeds display impairment of anti-oxidative mechanisms upon dehydration leading to oxidative damage and numerous lethal lesions (Finch-Savage et al., 1996). During storage, orthodox seeds have low moisture content with very little metabolic activity (Rajjou et al., 2012). In such conditions, detoxifying enzyme activity is negligible and therefore any build-up of ROS can lead to direct deleterious effects on nucleic acids, or can be trapped in the intracellular structures (Smirnoff, 1993) only to be released upon imbibition, causing damage to the genome. Further to this in numerous plant species, seed ageing is associated with a marked loss in antioxidant enzyme activity (Pukacka, 1991; De Vos et al., 1994; Bailly et al., 1996). Prolonged dry storage will exacerbate ROS accumulation as will storage in sub-optimal conditions such as high temperature and humidity (Bailly et al., 2004; Tian et al., 2008). This in turn means that older seeds are less able to cope with ROS associated damage as well as accumulating an increased amount of ROS based damage.

1.3.4 DNA strand breaks

Both single and double strand DNA breaks are primarily caused by oxidative damage, whereby the phosphodiester bond of one or both of the two strands are oxidised when high levels of ROS are present. SSBs are further generated as excision repair pathway intermediates in response to DNA damage by ROS (sections 1.4.3 and 1.4.4) (Bray and West, 2005). Strand breaks are also generated by radiomimetic compounds that imitate the effects of radiation, such as methyl methane sulphonate (MMS) and bleomycin. MMS is an alkylating agent that predominantly methylates DNA on the N3-deoxyadenosine and N7-deoxyguanosine of DNA bases, causing replication forks to stall during S-phase (Lundin et al., 2005). Bleomycin interacts with DNA and chelates heavy metal ions (e.g. Fe^{2+}), producing a pseudoenzyme that reacts with oxygen to produce superoxide and hydroxide free radicals that bind

and cleave DNA through attack of the phosphodiester bond (Claussen and Long, 1999).

Double strand breaks are the most severe type of DNA lesion. Unlike most other forms of DNA damage, both strands of the DNA double helix have a break in the phosphate backbone and therefore a complementary strand is not present to guide repair. If DSBs are not repaired they lead to chromosomal fragmentation and substantial loss of genetic information during cell division and are therefore highly toxic to the cell. DSBs can be induced directly by IR through simultaneous oxidation of both strands (Chen et al., 1996) and by bleomycin (Claussen and Long, 1999). Cells in S-phase of the cell cycle display increased levels of DSBs, caused by continued stalling of the replication fork when SSBs or a block on the template strand is encountered. The replication fork then ends up collapsing, generating a double strand end (Kuzminov, 2001). Programmed DSBs are also generated in cells during meiosis. Induction of DSBs is used to promote homology searching leading to the recombination and synapsis of homologous chromosomes during prophase I of meiosis (Grelon et al., 2001). This allows homologous recombination and facilitates the production of offspring diversity.

1.4 Repair of single strand damage of the DNA duplex

Plants display a greater resistance to DNA strand breaking genotoxins than humans, with maize (*Zea mays*) surviving X-ray doses two to three times the 10-Gray dose lethal to humans, even though they have comparable genome sizes (Killion and Constantin, 1972). Plant cells possess several pathways for the repair of DNA damage and specific DNA lesions require particular repair pathways. Many of the plant DNA repair pathways are strongly conserved among eukaryotes and are best characterised in yeast and mammalian model systems. However, our understanding of DNA repair in higher plants has increasingly developed over the past few decades, particularly in *Arabidopsis* thanks to its sequenced genome, online resources and availability of specific T-DNA knockout mutants lines (Alonso et al., 2003).

1.4.1 Direct reversal and photoreactivation

Cells are known to eliminate some types of DNA damage by chemical reversal. These mechanisms do not require a template, since the types of damage they counteract can occur in only one of the four bases. These are UV-induced damage products known as pyrimidine dimers (section 1.3.1). Direct reversal mechanisms are specific to the type of damage incurred and do not involve breakage of the phosphodiester backbone. Pyrimidine dimers can be repaired by photoreactivation pathways and require DNA photolyases (Sancar, 1994). Two photolyases characterised in *Arabidopsis* are specific for photoreactivation of either cyclobutane pyrimidine dimers (CPDs) or pyrimidine (6-4) pyrimidones (6-4PPs), the two major UV-B-induced photoproducts in DNA and are called CPD and 6-4PP photolyase respectively (Waterworth et al., 2002). Photolyase enzymes bind to the pyrimidine dimer photoproduct and in a light-dependent reaction catalyse the split of the pyrimidine dimer (Dany et al., 2001). Interestingly whilst photolyases are phylogenetically conserved in many species, from the bacteria, fungi, plants and animals, the photolyase activities are absent in humans and other placental mammals. Humans instead rely on a

different repair mechanism to correct pyrimidine dimers termed nucleotide excision repair (section 1.4.4) (Lucas-Lledo and Lynch, 2009).

1.4.2 Mismatch repair

The mismatch repair pathway repairs incorrect nucleotides which are mis-incorporated by DNA polymerase during DNA synthesis (DNA replication and recombination) or damaged bases resulting from base modification. It is one of the DNA repair pathways used in animals and plants to maintain genomic stability by targeting base–base mismatches and insertion/deletion loops. Mismatch repair also functions in homologous recombination, controlling the level of mismatches tolerated by the invading strand (Hays, 2002). Detection of mutated bases is performed by a heterodimer of MSH proteins. These excise the mis-incorporated bases and the resulting gap is filled with a DNA polymerase and a DNA ligase (Iyer et al., 2006). Several characterised MSH proteins have been identified in *Arabidopsis* and exhibit high conservation with their mammalian counterparts and have important roles in meiotic recombination (Higgins et al., 2008). A novel MSH protein has also been discovered in *Arabidopsis* and shows, along with 3 other MSH proteins, to form three distinct protein heterodimers with different specificities for mismatched DNA (Culligan and Hays, 2000).

1.4.3 Base excision repair (BER)

Base modifications are the most common type of endogenous DNA damage and in the mammalian genome accounts for thousands of lesions per day (Lindahl, 1993). The base excision repair (BER) pathway is very well conserved across all kingdoms, from bacteria to yeast, humans and plants (Kim and Wilson, 2012). It is likely BER evolved in response to the high level of spontaneous decay products that are formed in DNA as a coping mechanism to the damage created upon reactions with natural endogenous chemicals, most notably ROS. BER has been extensively studied in higher eukaryotes but it is best understood in mammalian systems. BER deals predominantly with small non-bulky nucleobase lesions, damaged bases (e.g. 8-oxoG) derived from oxidation, alkylation or deamination or excising and replacing incorrect bases (e.g. uracil)

(Barnes and Lindahl, 2004; Fortini and Dogliotti, 2007; Zharkov, 2008). All living organisms have specific enzymes involved in the base excision repair (BER) pathway and can occur throughout the cell cycle (Morita et al., 2010).

Multiple BER mechanisms exist but each pathway shares common elements and generally involve the following steps. The first part of all BER pathways is the recognition and removal of incorrect or damaged substrate bases. This is initiated by DNA glycosylases, which recognise and remove specific damaged or inappropriate bases forming AP sites to create an abasic site intermediate. An incision of the abasic site is then made by an apurinic/apyrimidinic (AP) lyase or endonuclease, the incision is in the 5' side of the AP site, leaving 3'-OH and 5'-deoxyribose-5-phosphate (5'-dRP) termini (Dianov et al., 1992). The remaining sugar fragment is removed by a phosphodiesterase or lyase. A DNA polymerase fills the gap left by the excised nucleotide and a DNA ligase generates a covalent phosphodiester bond between the 3'-OH end of the upstream nucleotide and the 5'-PO_4 end of the downstream nucleotide (Tomkinson et al., 2006). These are then cleaved by an AP endonuclease, such as ARP (Murphy et al., 2009). The resulting single-strand break can then be processed by either short-patch (where a single nucleotide is replaced) or long-patch BER (where 2-10 new nucleotides are synthesized) (Córdoba-Cañero et al., 2011).

1.4.4 Nucleotide excision repair (NER)

NER is the most well-studied mechanism for repairing UV-photoproducts in eukaryotic cells, in part because defects in human NER genes contribute to the photosensitive syndromes xeroderma pigmentosum (XP), Cockayne syndrome, and trichothiodystrophy (Kunz et al., 2005). NER involves damage recognition, incision of the damaged DNA strand on each side of the lesion, release of the lesion-carrying oligonucleotide, repair synthesis, and ligation to seal the repair patch. Recognition of DNA damage in the NER pathway is initiated in two distinct ways, which then use the same machinery to repair the damage. When damage occurs on the transcribed stranded of an actively expressing gene, transcription coupled repair (NER-TCR) removes it.

In the NER-TCR sub-pathway, NER repair machinery preferentially targets regions of the genome that are actively transcribed, this ensures interference of the lesions with transcription is avoided. This pathway is activated during transcription by stalling of elongating RNA POLYMERASE II (RNAPII) and is dependent on the recruitment of two proteins (COCKAYNE SYNDROME A and B) to the damaged site (Svejstrup, 2007). The other NER sub-pathway is known as global genome repair (NER-GGR). Lesions in this pathway are detected by the UV-damaged DNA-binding protein complex and another protein complex containing RAD23, XPC and centrin proteins (Volker et al., 2001).

Both sub-pathways subsequently require the TRANSCRIPTION FACTOR-IIH (TFIIH) complex, which unwinds the DNA helix in the locality of the damaged site. This DNA unwinding activity is associated with the conserved helicase motifs observed in the XERODERMA PIGMENTOSUM GROUP B (XPB) and XPD subunits of the TFIIH complex. EXCISION REPAIR CROSS-COMPLEMENTING PROTEIN 1 (ERCC1), XPF and XPG are structure-specific endonucleases that excise DNA around the damage site. Following excision of the lesion, the oligonucleotide containing the damaged site is released, DNA repair synthesis (made by DNA polymerase δ or ε) and the ligation reaction occur, thus completing the NER process (Sancar and Tang, 1993)

1.5 Repair of DNA double strand breaks

In all organisms all components of DNA are susceptible to damage i.e. the purine and pyrimidine bases, the phosphodiester linkages and the sugar residues all can incur damage. Many different genotoxic stresses originating from both in and outside of the a plant cell can cause DSBs. Environmental agents such as UV light, ozone and other reactive oxygen species, desiccation and rehydration as well as air and soil pollutants such as heavy metals can cause DSBs (Waterworth et al., 2011). Inside the cell, spontaneous DSBs can occur resulting from DNA replication defects, exemplified by collapsed replication forks, steric stresses during DNA unwinding, during replication and replication past a site of DNA damage (Kuzminov, 2001). DSBs pose a severe threat to the genome integrity of plants throughout their lifecycle. In yeast just one DSB in a cell can potentially cause a loss of viability in that cell, however, plants are thought to have a higher tolerance (Waterworth et al., 2011). In contrast to the serious threats posed by DSBs, programmed initiation of such DNA lesions play integral roles in early stage gamete formation, facilitating chromosome pairing and homologous recombination associated with meiosis (Schuermann et al., 2005).

The result of many different stresses that can cause DSBs means marked heterogeneity in the DSB products is created. Even if lesions occur at identical sequences of DNA the structures of those DSBs formed may be entirely different. DSB products often lack the 3’ hydroxyl and 5’ phosphate groups usually associated at naked ends of DNA when produced *in vitro* by restriction enzymes, creating challenges in the repair of such lesions [Figure 1-4]. A well conserved family of proteins known as the DNA ligases are responsible for chemically re-joining (re-ligation) the two ends back together after a DSB has occurred. Three such proteins have been identified in the plant *Arabidopsis thaliana*; DNA LIGASE 1 (LIGI), DNA LIGASE 4 (LIG4) and DNA LIGASE 6 (LIG6). Homologues of LIG1 and LIG4 exist in fungi and mammals but LIG6 is only found in plant species (Taylor et al., 1998; West et al., 2000; Waterworth et al., 2010). Ligase enzymes require 3’ hydroxyl and 5’ phosphate groups at the ends

of DNA strands to facilitate re-ligation (Bray et al., 2008). A range of DNA end processing factors, modifying DNA ends making them suitable for re-ligation by DNA ligases have been identified in yeast and mammals (Lieber, 2010). Such factors are poorly understood in plants.

This marked heterogeneity in DSB products creates further problems for cells; a single DSB is potentially a lethal event and can severely inhibit plant growth and development. It is therefore important that plant cells have a multitude of DNA damage signalling and repair mechanisms to ensure quick, accurate and effective repair of DSBs.

1.5.1 DNA double strand break detection and signalling

The potentially lethal consequence of DSBs underpins how essential early detection and accurate repair is in plants. Cell cycle arrest allows the time needed for the cell complete this DNA repair, but still requires rapid detection to dampen any detrimental effects (Waterworth et al., 2011). Several proteins have been identified in *Arabidopsis* as candidates for the detection of DSB including KU70 and KU80 (Charbonnel et al., 2010). Homologues with conserved function of these proteins were first identified in yeast through *in vitro* assays and demonstrated the complex has a high affinity for exposed DNA ends (Lieber, 2010). KU70 and KU80 homologues are well characterised in yeast and mammals and are key components in DSB detection (Lieber, 2010). In *Arabidopsis*, the KU complex is a key component of one DSB repair pathway, the so called non-homologous end joining pathway (NHEJ) (West et al., 2004).

In the absence of the KU70/KU80 complex, microhomology is utilised to physically join the two ends of a DNA DSB event before the MRE11-RAD50-NBS1 (MRN) complex facilitates chemical re-ligation of the two ends. The MRN complex has further roles in facilitating the DNA damage response. It has been shown in yeast and *Xenopus* that NBS1 recruits the protein kinase ATM (You et al., 2005). Upon recruitment, kinase activity is strongly up-regulated, directly or indirectly facilitating a multitude of cellular changes associated with DNA damage repair responses (Section 1.6.1)

Once a DSB has been detected and an initial repair response has occurred, there is a choice of repair mechanisms available to a plant. Two major classes of DSB repair pathway exist, as in all eukaryotes: homology-dependent and homology-independent repair pathways (Waterworth et al., 2011). Homology-dependent recombination, otherwise known as homologous recombination (HR), requires the presence of an identical or nearly identical sequence to be used as a template. DSB repair by this pathway can lead to major changes in the genome through deletions, insertions, chromosome translocations and inversions (Mazon et al., 2010). HR can mediate accurate repair which does not lead to the production of any mutations within the genome (section 1.5.2). The second repair pathway is known as the non-homologous end joining pathway (NHEJ). It does not rely on the presence of a homologous DNA strand but instead joins the two strands of DNA with exposed ends together (section 1.5.2). In contrast to the HR pathway, NHEJ is intrinsically mutagenic and so will typically lead to at least small changes in DNA sequence local to a DSB event (Osman et al., 2011).

1.5.2 Homologous Recombination

Homologous recombination relies on regions of homology and base-pairing between two different DNA duplexes. Different pathways of HR repair have been demonstrated in plants, termed single-strand annealing (SSA) [Figure 1-6], synthesis-dependent strand annealing (SDSA) and double strand break repair model (DSBR) [Figure 1-7].

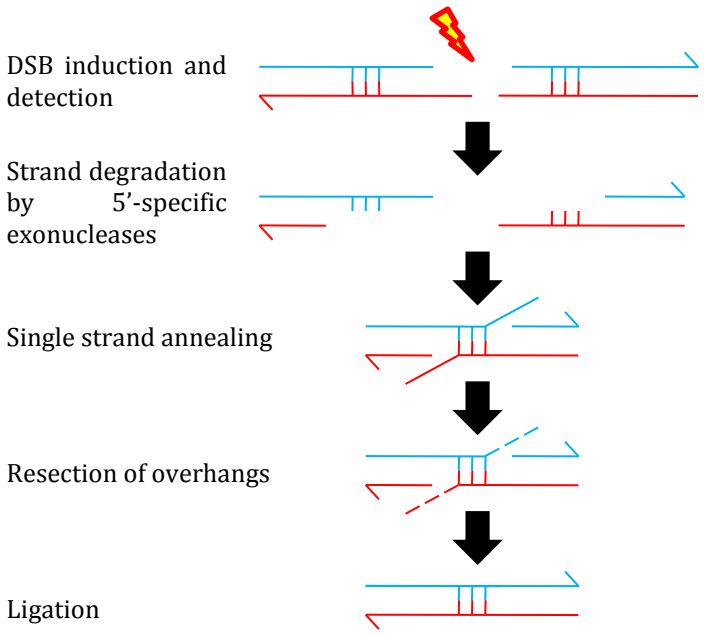


Figure 1-6 Single Strand Annealing (SSA)

The SSA pathway does not require a similar or identical separate molecule of DNA, unlike SDSA or DSBR pathways, making it unique in with regards to HR. The SSA pathway utilises one single DNA duplex instead and repeat sequences within it provides the identical sequences that homologous recombination needs for repair.

The first, SSA has been shown to be the most common HR pathway active in plants when a DSB occurs between two repeated sequences (Siebert and Puchta, 2002). It accounts for up to one third of repair events in such circumstances, however, this pathway is non-conservative and results in the deletion of any intervening sequence (Mazon et al., 2010). The latter two pathways share a common beginning before diverging: at the site of a DSB, long 3' single-stranded DNA ends are produced by the exonuclease activity. The newly formed ssDNA then probes for matching strands on the homologous chromosome or sister chromatid (Edlinger and Schlogelhofer, 2011). When a site of homology has been found, DNA synthesis can proceed using the homologous region as a template. The opposite strand of the invaded duplex is displaced to allow further strand invasion and branch migration. It is at this point the SDSA and DSBR pathways diverge [Figure 1-7].

In the SDSA pathway, the invading strand recombines with the original chromatid after dissociation from the invaded duplex (Waterworth et al.,

2011). A mechanistically different process to SDSA can occur during the 3' end extension if DNA synthesis continues all the way to the end of the chromosome, this process is referred to as break-induced replication (BIR) (Llorente et al., 2008). Work in the field bean, *Vivia faba*, in which simultaneous incorporation of ethynyldeoxyuridine (a base analogue) and DSB induction resulted in no induction of DNA replication, suggesting that in higher plants BIR does not play a major role in DSB repair (Schubert et al., 2011).

The displacement of one strand in the invaded duplex during strand invasion leads to the formation of the D-loop [Figure 1-7]. In the DSBR model, second end capture occurs where the displaced strand base-pairs with the homologous region of the 3' end on the other side of the original break. The chromatids become linked together by the formation of two Holliday junctions (Osman et al., 2011). The resolution of the Holliday junctions occurs in one of two ways, either crossover between chromosomes will occur or not, this is dependent on where the DNA strands are cut (Mazon et al., 2010).

One of the important features common to all the HR pathways is the search for homology preceding strand invasion. *In vitro*, just one protein is required for this step, termed RAD51, which has an essential role in HR in vegetative tissues. Further to this, in *Arabidopsis,* RAD51 knockout mutants are sterile (Li et al., 2004) but are otherwise phenotypically normal. Mutant *rad51* plants do not display hypersensitivity when treated with DSB-inducing agents (Li et al., 2004). This implicates a relatively minor role for HR in double strand break repair in comparison to NHEJ (section 1.5.3). In contrast to *Arabidopsis* and higher plants, the moss, *Physcomitrella patens*, utilises HR as the major pathway for double strand break repair (Markmann-Mulisch et al., 2007). In addition to RAD51, many other proteins working in distinct complexes are required to process strand invasion and subsequent intermediates of recombination. Additional factors include XRCC2, XRCC3 and RAD51-like proteins (RAD51B, RAD51C and RAD51D) (Osman et al., 2011). Further factors which aid homology searching include the DNA helicases and chromatin-remodelling enzymes (Blanck et al., 2009).

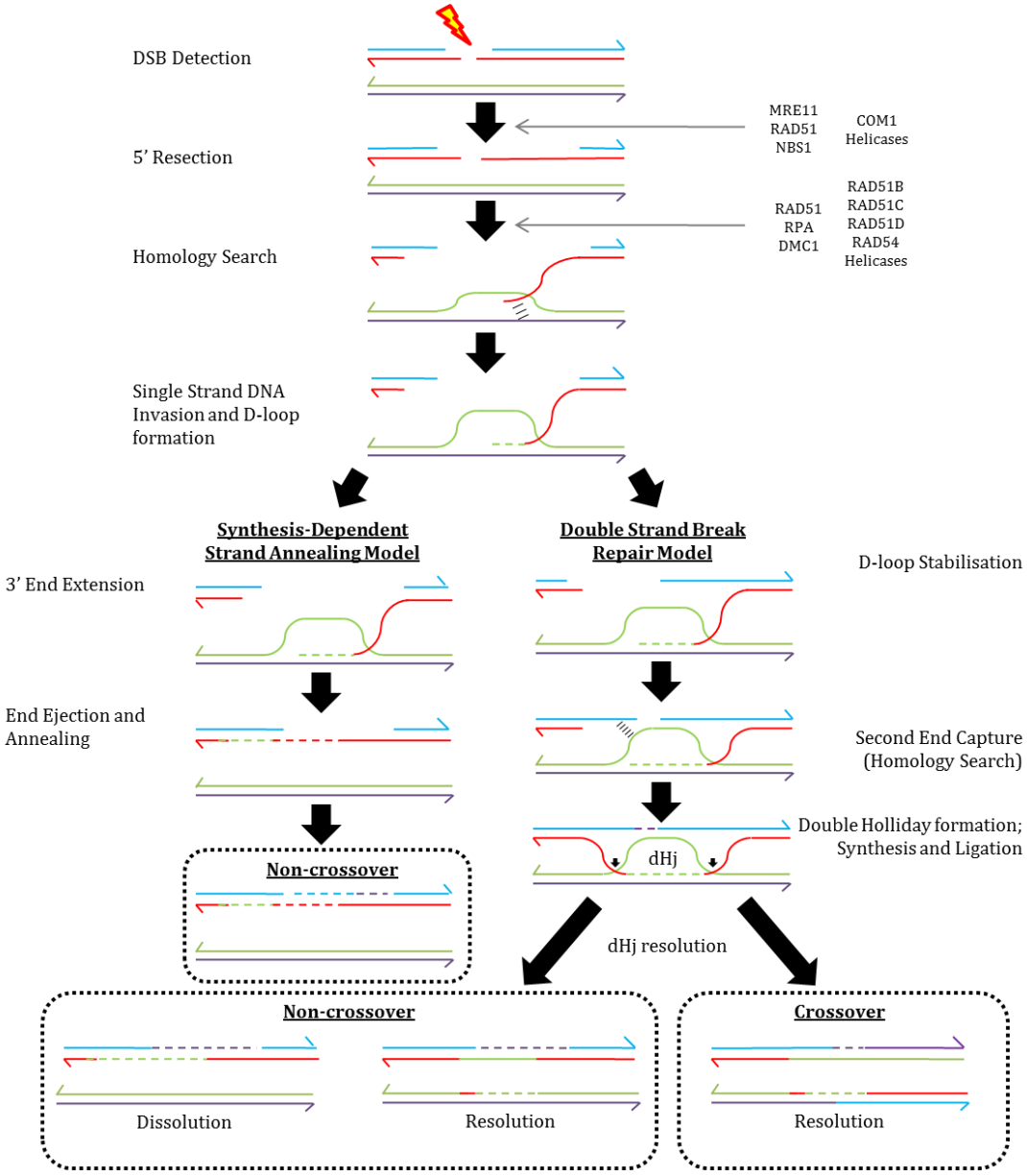


Figure 1-7 Synthesis-Dependent Strand Annealing (SDSA) and Double Strand Break Repair Model (DSBR) of homologous recombination

Several key recombination pathway proteins are shown at the stage at which they are active. After the initial strand invasion, D-loop formation and DNA synthesis has begun, the SDSA and DSBR pathways diverge. SDSA is thought to occur during S-phase when a sister chromatid has been synthesised and can be used as a template. The DSBR pathway occurs in meiosis and facilitates chromosome crossovers in creation of genetically diverse gametes. Crossovers are much rarer in SDSA in comparison to DSBR.

The highly repetitive nature of the genomes of higher eukaryotes has implications for the ability to utilise homologous recombination as a route of DSB repair (de Koning et al., 2011). Inappropriate recombination between two repetitive elements found at different loci within a genome has the potential to cause lethal mutations and so HR activity must be carefully regulated. In *Saccharomyces cerevisiae,* RAD51 has been shown to be regulated at the protein level by RAD52 (Liu and Heyer, 2011). This protein promotes homologous recombination by facilitating the nucleation of the RAD51 recombinase onto pre-processed single-stranded DNA. A RAD52 homologue is found in mammalian genomes but knockouts in mice display very little abnormal phenotypes with regards to repair, recombination or meiosis. Instead, BRCA2, a protein with no homologue in *Saccharomyces cerevisiae* maintains this central HR function in mammals (Liu and Heyer, 2011). Whilst it has been established a loss RAD52 function has no impact on cell growth and viability in human cells with functioning BRCA2 activity, recent reports show a loss in functional Rad52 is synthetically lethal where cells are already BRCA2 deficient (Feng et al., 2011). This implicates a more important role for the function of Rad52 in vertebrates than previously thought. A BRCA2 homologue in plants has been identified and characterised to have the same RAD51-binding activity as its mammalian counterpart and has importance in DNA repair (Abe et al., 2009). Two RAD52 homologues have been reported in *Arabidopsis* and RNA interference lines defective for both copies show an increased sensitivity to mitomycin C treatment and reduced levels of fertility (between 25-50%) (Samach et al., 2011).

Evidence suggests regulation of HR activity occurs in a cell cycle-dependent manner. Work in yeast has shown HR is up-regulated in S and G2 phase during which times a sister chromatid is available as a template for repair (Huertas et al., 2008). The production of 3’ single stranded ends by 5’ resection is an important aspect in this regulation; Com1/Sae2 has been shown to control DNA-end resection in *Saccharomyces cerevisiae* (Huertas et al., 2008). CtIP (a homologue of Com1/Sae2) in *Xenopus* has been found to perform a similar role as well as interacting with the MRN complex (Com1/Sae2 interacts with the

homologous MRE11-RAD50-XRS2 (MRX) complex in *Saccharomyces cerevisiae*). Furthermore, in both yeast and vertebrates activity of Com1/Sae2 and CtIP is regulated by CDK-mediated phosphorylation at consensus CDK sites (Huertas et al., 2008; Wang et al., 2012). A homologue in plants has been described and it is possible the *Arabidopsis* COM1 protein regulates HR in a similar way to that demonstrated in yeast and vertebrates (Uanschou et al., 2007; Yun and Hiom, 2009).

1.5.3 Non-homologous end joining

1.5.3.1 The canonical non-homologous end joining pathway

Whilst mutations in genes required for NHEJ in mammals have often been found to be lethal, equivalent *Arabidopsis* knockout genes have a wild-type phenotype under normal growth conditions. However, if these mutants are exposed to high levels of genotoxic stress, knockouts often show growth hypersensitivity relative to wild-type. The DNA damage response is found to be permanently activated, with increased incidence of cell death even in the absence of genotoxins (Fulcher and Sablowski, 2009). Conversely, little is known about NHEJ processes in plants relative to our knowledge of the pathways in mammals. Well characterised KU-mediated recruitment of polymerases, end-processing accessory factors and even nuclease activities in mammals has so far not been translated into a similar characterisation in plants. This is due to the fact that many of these factors lack characterised homologues in plants, assuming they exist at all (Lieber, 2010). An exception is DNA POLYMERASE LAMBDA (POL λ) in *Arabidopsis,* which are mildly sensitive to DNA double strand breaks but defective in integration of a transgene. Double *ku70 polλ* mutants do not exhibit increased sensitivity to single *ku70* lines, consistent with function through the same repair pathway as *KU70* (Furukawa et al., 2015).

The KU70-KU80 complex is a central component of the NHEJ pathway of DSB repair. The Ku complex in mammals is able to recognise, with high affinity, exposed ends of DNA by virtue of its ring-like structure (Grundy et al., 2014). Stabilising and protecting the broken DNA ends from exonuclease-mediated

degradation, the Ku complex then recruits the DNA-dependent protein kinase, DNA-PKcs, which is activated upon association with DNA. Subsequent autophosphorylation and phosphorylation of many other proteins initiates downstream end processing and ligation. One target of DNA-PKcs-mediated phosphorylation is the ARTEMIS nuclease upon which becomes active and opens and trims back DNA creating over-hanging strands in DNA hairpins in the preparation of ligation (Lieber, 2010). No homologues of either DNA-PKcs or Artemis have been found in yeasts or plants. Instead the Ku complex has been shown to interact with the MRX complex and DNA ligase IV to bring about NHEJ in *Saccharomyces cerevisiae* (Lieber, 2010).

1.5.3.2 Back-up non-homologous end joining pathways

A highly diverse range of stresses cause DSBs in DNA, as a result there is a marked heterogeneity in the DSB structures produced. The NHEJ pathway has evolved to process a wide range of repair substrates (Lieber, 2010). In mammals many different sub-pathways of NHEJ have been described. Flexibility is highlighted by the well-defined classical (or canonical) NHEJ (c-NHEJ) pathway. This pathway can undergo extensive rounds of enzymatic activity which, if unsuccessful, can recruit other repair factors (Lieber, 2010; Waterworth et al., 2011). Sub-pathways known as the backup NHEJ pathways (B-NHEJ) include alternative end joining (alt-EJ) and microhomology-mediated end joining (MMEJ). Alt-EJ and MMEJ are Ku-independent and *Arabidopsis ku70* and *ku80* mutants display an increased incidence of these B-NHEJ pathways (Heacock et al., 2004). Furthermore MRE11, from the MRN complex, is required for MMEJ activity as observed in *Arabidopsis ku70* mutants (Heacock et al., 2004) (Figure 1-8).

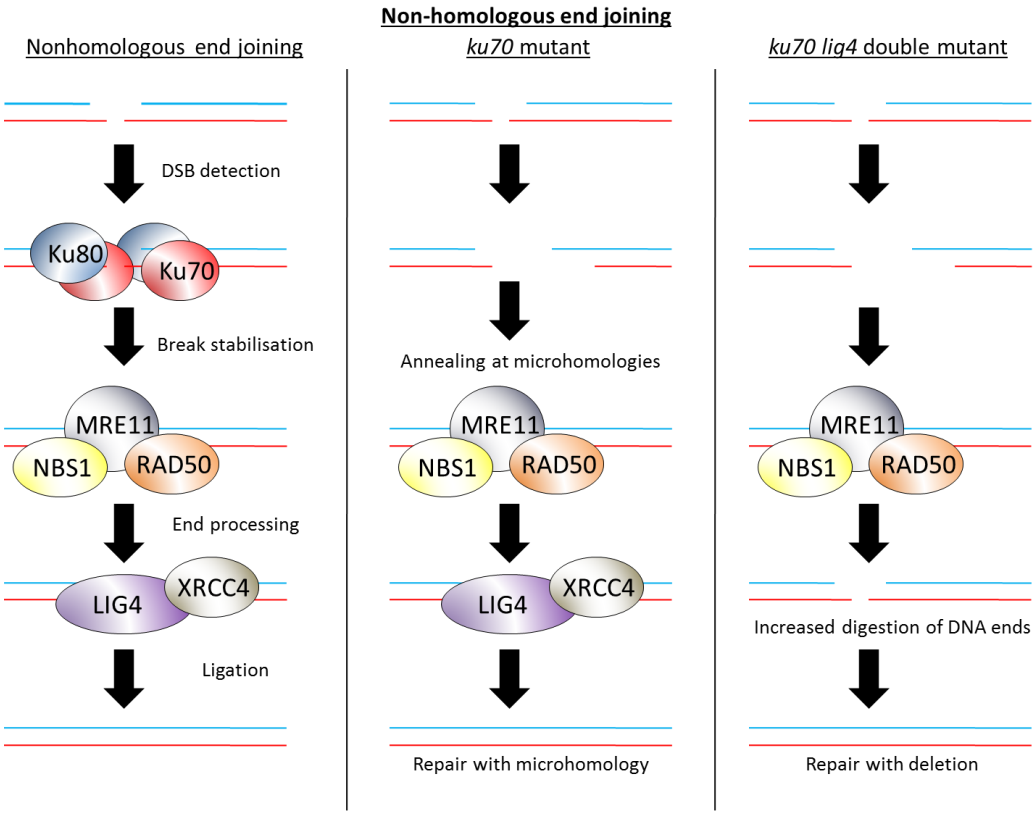


Figure 1-8 Pathway of the canonical and alternative nonhomologous end joining in plants.

The conserved nonhomologous end-joining (NHEJ) pathway is mediated by the KU70–KU80 complex, which possesses DNA end-binding activity. The MRE11, RAD50 and NBS1 (MRN) complex has double-strand break (DSB) detection, signalling, end-processing and structural roles. The DNA ligase activity of the DNA LIGASE 4 (LIG4)–XRCC4 complex seals the phosphodiester backbone. End joining can occur in the absence of KU; this repair pathway displays greater dependence on microhomologies and requires the presence of either MRE11 or LIG4. Adapted from (Waterworth et al., 2011)

1.6 Responses to DNA damage in plants

Responses to DNA damage are controlled by two master phosphoinositide-3-kinase-related protein kinases (PIKKs) in plants. ATAXIA TELANGIECTASIA MUTATED (ATM) and ATAXIA TELANGIECTASIA AND RAD3 RELATED (ATR) have conserved functions in eukaryotes coordinating cell cycle arrest, transcriptional responses and chromatin remodelling for subsequent progression of the DNA repair pathways (Garcia et al., 2003; Culligan and Britt, 2008; Drury et al., 2012) (Figure 1-9).

1.6.1 The role of ATM and ATR

There is some overlap in the function and responses of ATM and ATR to DNA damage, as either is sufficient for the observed down-regulation of a subset of transcripts following irradiation. Partial redundancy of ATM and ATR is also observed in the meiotic phenotypes of single and double mutants; *atr* displays full fertility, whilst *atm* shows only 10% that of wild-type, whereas the double mutant is completely infertile (Culligan and Britt, 2008). However, ATM is predominantly activated by DSBs and activates downstream signalling pathways leading to DNA repair, transient arrest of the cell cycle and inhibition of DNA replication (Culligan and Britt, 2008). ATR shows stronger activation by regions of single-stranded DNA associated with replication defects (Culligan et al., 2006). These can occur either spontaneously, after genotoxic stress, UV irradiation or hydroxyurea exposure, a chemical that causes DNA replication defects by inhibiting the enzyme ribonucleotide reductase, depleting the dNTP pool.

In mammals, ATR and ATM directly and indirectly phosphorylate many hundreds of target proteins including the checkpoint associated protein kinases Chk1 and Chk2, Nbs1 and the histone 2A isoform H2AX (Matsuoka et al., 2007). For a long time, the majority of signalling components downstream of these two kinases had remained uncharacterised in plant species. One protein that has been shown to be phosphorylated in an ATM-dependant manner is SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1) [Figure 1–8], a plant-specific

transcription factor that plays a central role in the DDR (Yoshiyama et al., 2009). Interestingly, the characteristics of SOG1 are similar to those of mammalian p53, even though the proteins' amino acid sequences are unrelated (Yoshiyama et al., 2014). Another exception has been the highly conserved rapid ATM-dependent phosphorylation of the histone variant H2AX in response to DSB (Friesner et al., 2005). Recent phosphoproteomic work in *Arabidopsis* has begun to identify more phosphorylation targets; these include the NHEJ factor LIG4 and DNA damage response element MRE11 of either ATM or ATR. Other targets include HTA10 and ASK1, involved in meiosis and WAPL, PDS5 and PCNA1 implicated in DNA replication (Roitinger et al., 2015). In the root and shoot meristems of *Arabidopsis*, ATM is also involved in initiating programmed cell death (PCD) to safeguard genome integrity in their stem cell populations (Fulcher and Sablowski, 2009).

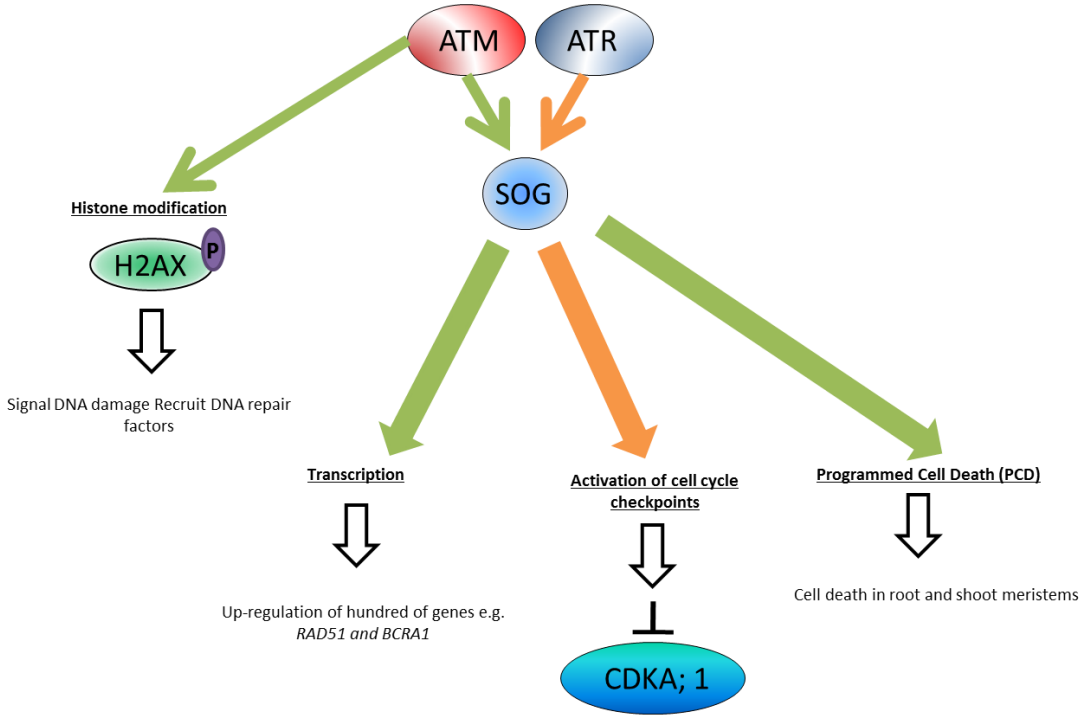


Figure 1-9 ATM/ATR-mediated responses to DSBs

ATM and ATR are both activated by DNA damage. There is some overlapping function but ATM is activated principally by DSBs and ATR has strong activation in response to ssDNA when replication at a replication fork is blocked. The ATM/ATR-mediated DNA damage response induces a large variety of cellular activities including those shown in this figure. Green arrows indicate ATM-mediation plays a major role in eliciting that cellular response. Orange arrows represent those in which ATR plays the major role in mediating a response. Indirect or downstream effects are indicated by open arrows and inhibition by a flat ended line. SOG1 is involved in many aspects of the ATM/ATR signalling pathways. ATM-dependant phosphorylation of histone H2AX aids in the recruitment of DNA repair factors. ATM is responsible for most transcriptional changes and regulation of programmed cell death (PCR). ATR activates cell cycle checkpoints; one example in plants is the ATR-mediated activation of WEE1 a protein kinase which in turn phosphorylates CDKA;1. This phosphorylation inactivates CDKA;1 kinase activity required for cell cycle progression leading to arrest. Both ATM and ATR activation can cause endocycle induction (Adachi et al., 2011). ATM and ATR are known to directly phosphorylate histone H2AX around the sites of DSB damage associated DNA repair (Friesner et al., 2005).

1.6.2 Transcriptional changes in response to DNA damage

The transcriptional response of genotoxic stress is integral to the DNA damage response of *Arabidopsis*. The subset of induced transcripts is poorly conserved between kingdoms, as such, work in mammalian and yeast systems cannot be translated to plants. The major controller of the transcriptional DNA damage response is ATM in plants, although ATR does have a minor role in gene expression regulation (Culligan et al., 2006). The ATM-mediated transcriptional response to DNA damage is highly specific to DSBs (Molinier et al., 2005) and is characterised by the substantial up-regulation of hundreds of genes, many displaying over a hundred-fold induction after a 100-Gy γ-ray dose. Many of the genes up-regulated by ATM and ATR are involved in DNA repair processes, cell cycle control and DNA metabolism. These include the homologous recombination factor *RAD51* and the endonuclease *COM1*/*CtIP*, components involved in DNA synthesis and the DNA signalling factors *RAD17, BREAST CANCER ASSOCIATED-GENE 1* (*BRCA1*) and *POLY ADP-RIBOSE POLYMERASE* (*PARP*) (Culligan et al., 2006). Interestingly, in *Arabidopsis* vegetative tissue there is little transcriptional change of the NHEJ-associated genes in response to DSBs (Culligan et al., 2006). Some genes are down regulated in response to DNA damage; including some expressed in G2 and M phase of the cell cycle (section 1.6.3).

1.6.3 DNA damage checkpoints and cell cycle

The DNA damage response and control of cell cycle progression is closely linked to the activation and release of checkpoints, which regulate entry and progression through the cell cycle. Plants deficient in checkpoint genes progress through the cell cycle in the presence of DNA damage. This ultimately leads to hypersensitivity to genotoxins and the suppression of growth. An example of this is in *atr*-deficient *Arabidopsis* seedlings, which display poor growth when treated with hydroxyurea (Culligan et al., 2004).

In eukaryotes, the core cell cycle machinery is well conserved and controlled by complexes of cyclin and cyclin-dependent kinases (CDKs) (Dewitte and Murray, 2003). Plants contain multiple CDKs, including CDKA;1 that are involved in the

regulation of the G1/S transition and G2. CDKA;1 contains regulatory phosphorylation sites including at Thr-14 and Tyr-15 which play key roles in controlling cell cycle by the activities of protein kinases and phosphatases (Inze and De Veylder, 2006). The protein kinase WEE1 controls the phosphorylation of CDKA;1 in plants. When exposed to hydroxyurea, *wee1*-deficient plants fail to undergo cell cycle arrest and conversely WEE1 overexpression causes phenotypes consistent with permanent activation of cell cycle checkpoints, including cell cycle arrest, differentiation of stem cells and resulting shrinkage of the meristem (De Schutter et al., 2007). These results collectively point to a key role for WEE1-mediated CDKA;1 phosphorylation in the control of the plant cell cycle in response to replication stress. However, WEE1 is not required for the response to DNA damage or for normal cell cycle progression (Cools et al., 2011). The nature of DNA damage checkpoints in plants remains to be fully elucidated.

In response to DNA damage, transcripts encoding the G2-associated cyclins and cyclin dependent kinases (CDKs) CYCB1;2, CDKB1;2 and CDKB2;1 are down-regulated within 8 hours of γ-rays. Reduced expression continues for 24 hours subsequent to DNA damage (Culligan et al., 2006). The G2/M phase-specific CYCB1;1 transcripts exhibits rapid induction within 1 hour of DNA damage in an ATM-dependent manner, which peaks around 8 hours, finally reducing by 24 hours. However, a GUS promoter and partial translational CYCB1;1 fusion shows slower kinetics and greater dependence on ATR, inciting differential responses between transcript and protein (Culligan et al. 2006).

1.6.4 Deoxyribonucleotide synthesis for DNA repair and cell cycle

With the notable exception of direct reversal, nearly all DNA repair pathways require DNA repair synthesis processes to operate. When single strand breaks occur, the repair machinery removes nucleotides around the area of damage and fresh deoxyribonucleotides (dNTPs) are required for synthesis of a new strand of DNA. In DSB repair new strands of DNA may be synthesised, catalysed by DNA polymerases after resection around the double strand break site

(Waterworth et al., 2011). DNA ligases are then responsible for joining the newly synthesised oligonucleotide to the 5' side of resected DNA (Bray et al., 2008). Synthesis of deoxyribonucleosides and deoxyribonucleotides can occur via *de novo* synthesis or salvage pathways.

Ribonucleotide reductase (RNR) is an essential enzyme that provides dNTPs for synthesis of DNA via the *de novo* pathway. RNR proteins comprise two large (R1) and two small (R2) subunits, which form the protein that catalyses a rate-limiting step in DNA precursor synthesis (Kolberg et al., 2004). The R2 subunit houses the di-iron tyrosyl radical cofactor essential for the reduction of ribonucleotide diphosphates (NDPs) to deoxyribonucleosides (dNDPs). Hydroxyurea is a well-known chemotherapeutic drug that reversibly binds to and inhibits the R2 subunit by acting as a scavenger of the tyrosyl free radical. The R1 subunit is responsible for binding of the nucleoside diphosphate substrates along with allosteric effectors and is the target feedback regulation to ensure appropriate production levels of dNTPs, ensuring adequate NDP units are left for RNA synthesis (Elledge et al., 1992; Kolberg et al., 2004). Studies in yeast and mammals have shown that defective RNR often led to cell cycle arrest, growth retardation, and in mammals, p53-dependent apoptosis, whereas abnormally increased RNR activities leads to higher mutation rates (Elledge et al., 1992; Chabes et al., 2003). Three *R2* genes in *Arabidopsis* have been characterised; *RNR2A, RNR2B* and *TSO2*. The expression of *TSO2* is increased proportionally upon exposure to DNA damaging agents and is part of the DNA damage transcriptional response. Further to this, *tso2 rnr2a* double mutants are found to be more sensitive to UV-C light with seedlings exhibiting increased DNA damage when compared to wild-type (Wang and Liu, 2006; Roa et al., 2009).

A second pathway exists to supply the cell with nucleic acid precursors. This salvage pathway is controlled by deoxyribonucleoside kinases (dNKs) which phosphorylate deoxyribonucleosides into the corresponding 5'-monophosphate deoxyribonucleosides. These enzymes are well characterised in eukaryotes from unicellular organisms to insects and mammals, but

interestingly these enzymes are not present in yeast and other fungi. Different kinases are found in different organisms, in *Dictyostelium discoideum*, an amoeba, three dNKs are found: deoxyguanosine kinase, deoxyadenosine kinase and thymidine kinase 1 (TK1) (Sandrini et al., 2007). In the fruit fly, *Drosophila melanogaster,* one gene has been identified that encodes a multi-substrate enzyme that has four dNK activities (Munch-Petersen et al., 1998), whilst humans have four different dNKs genes: TK1, deoxycytidine kinase (dCK), thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) (Eriksson et al., 2002). In recent years two TK1-like proteins: *TK1a* and *TK1b* and a further *dNK* gene with deoxyadenosine, deoxyguanosine and deoxycytidine kinase activities have been characterised in *Arabidopsis* (Clausen et al., 2012). It was thought that *TK1a* and *TK1b* have redundant roles, but recent work has shown *TK1a* transcription is up-regulated in response to UV-C irradiation, whilst *TK1b* remains unaffected. This suggests a specific role for *TK1a* in responding to DNA damage. Further to this *TK1a* over-expression lines in *Arabidopsis* demonstrate an decreased tolerance to DSBs, as shown by hypersensitivity to MMC and zeocin, a DNA intercalator which induces DNA DSBs (Pedroza-García et al., 2014).

1.7 DNA damage and repair in seeds

In plants, genomic integrity is preserved through the co-ordinated action of different DNA repair pathways. Understanding of DNA repair mechanisms *in planta* has dramatically increased in recent years with increased attention toward the molecular responses induced by environmental stresses (Kimura and Sakaguchi, 2006; Waterworth et al., 2011). However, the networks of DNA damage sensing and response functions have been only partially elucidated in plants.

In orthodox seeds, metabolism virtually ceases in the quiescent embryo and the low activity of cellular maintenance pathways, including DNA repair, results in a decline in seed viability during storage. In addition, dehydration, dry storage and subsequent water imbibition upon germination of the seed are associated with high levels of oxidative stress and significant damage to the integrity of the genome of the embryo of the seed. The latter are especially true if seeds are stored under unfavourable conditions such as high temperatures and moisture contents. By identifying important DNA repair components in seed germination potential targets may be uncovered for the generation of crops with improved seed storability and germination capacity. However, most of our current understanding of plant DNA repair has originated from studies using non-physiological stresses.

A number of pre-genomic era studies established a correlation between seed ageing and accumulation of DNA damage. The integrity of DNA was shown to be decreased in seed lots of lower viability (Cheah and Osborne, 1978b), and low quality seeds were more likely to produce abnormal seedlings than seeds of high quality (Roberts, 1972a). Deterioration of DNA quality in aged seeds was observed in rye embryos. The integrity of DNA was shown to be decreased in seed lots with lower viability (Cheah and Osborne, 1978b). Similarly in barley, broad beans and peas during storage there is a strong correlation between the loss of viability of seeds in storage, chromosomal breakages and the incidence of aberrant chromosomes (Abdalla and Roberts, 1969). Even in high-quality

seeds there are background levels of DSBs and '*there is no threshold level of viability before chromosomal aberrations occur*' (Dourado and Roberts, 1984).

The genomic revolution has allowed study of individual pathways by enabling the production of single gene knock outs and in recent years this has allowed further elucidation of the major DNA repair pathways. The different DNA repair pathways play specific roles which are important to ensure genome stability. However, little is known about the roles of each pathway with regards to seed germination.

1.7.1 DNA double strand break repair

The correlation between low quality seeds and DNA damage (Roberts, 1972b) suggests DNA repair pathways play an integral role in germination and in the longevity of stored seeds. Specific genes and pathways have been identified in these DNA repair processes which are important in maintaining high quality seeds.

Consistent with the observation that DSBs occur in high quality seeds during germination (Dourado and Roberts, 1984), unaged *Arabidopsis* seeds display increased transcriptional regulation of DSB-associated genes in the earliest stages of germination [Figure 1-10], coincident with DSB DNA repair synthesis (section 1.7.4.3) (Waterworth et al., 2010).

1.7.1.1 Homologous Recombination

Very little work has been reported linking HR to seed quality. When the *RAD51* homologue in maize was knocked-out, germination was delayed by 3 to 4 days after irradiation treatment and seedlings did not survive past 2 weeks whilst wild-type siblings survived to develop into apparently healthy seedlings (Li et al., 2007a).

1.7.1.2 Non-homologous end joining

NHEJ is important in the repair of DSBs during germination with irradiated *ku70* and *lig4* deficient seeds germinating more slowly than wild-type (Friesner and Britt, 2003). Work in *Arabidopsis* using *lig4* and *lig6* mutants importantly demonstrated the first molecular link between DNA damage and seed quality.

LIG6 is a DNA ligase unique to plants and is important for germination and viability, especially in aged seeds (Waterworth et al. 2010). *LIG4* is involved in the NHEJ pathway (section 1.5.3) and also involved in maintaining seed quality. Accelerated ageing procedures reduce both germination vigour and the viability in *lig6 lig4* double mutant seed lines, these are more severely affected by accelerated ageing protocols than the single *lig4* and *lig6* mutant lines (Waterworth et al., 2010). This suggests that these two ligases function in distinct repair pathways to remove DNA damage in seeds early in imbibition, possibly through the classical NHEJ (LIG4) and an alternative NHEJ pathway (LIG6).

1.7.1.3 DNA double strand break transcriptional response

In *Arabidopsis*, transcription of genes involved in the DNA damage response are significantly up-regulated by the presence of DSBs (section 1.6.2). Within 3 hours of imbibition, DNA damage response genes are also up regulated, these include *PARP2, RAD51, TK1a, TSO2* and *XRI1* [Figure 1-10]. After an initial steep rise in transcript levels, they begin and continue to fall as germination continues. Transcript levels then sharply increase nearer to the completion of germination with the subsequent synthesis of DNA related to cell cycle progression (Waterworth et al., 2010).

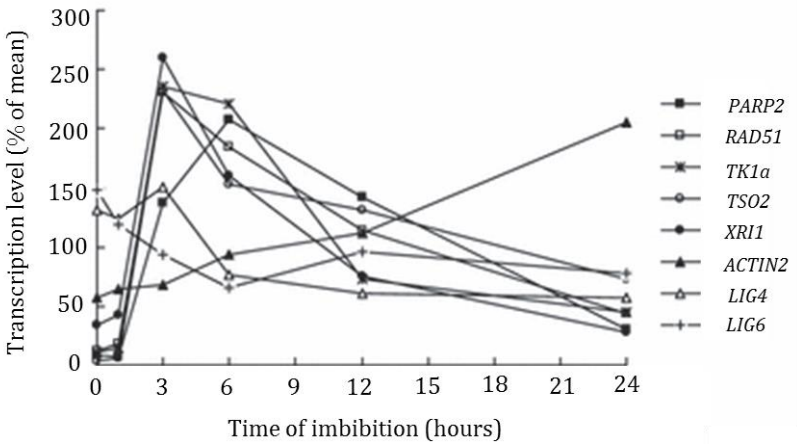


Figure 1-10 Activation of the double-strand break (DSB) transcriptional response in imbibed seeds.

Transient transcriptional activation of DSB-responsive genes during imbibition between 0 and 24 hours. This graph demonstrates that DSB detection is occurring very early with transcripts sharply rising and peaking at 3 hours post seed imbibition. Adapted from (Waterworth et al., 2010).

Work in the legume, *Medicago truncatula,* has also shown that the expression profile of some genes related to DNA repair is up-regulated when seeds were imbibed with PEG6000, these include *MtTDP1* (tyrosyl-DNA phosphodiesterase), *MtTOP1* (DNA topoisomerase I), *MtTFIIS* (transcription elongation factor II-S) and *MtTFIIS-like* (Balestrazzi et al., 2011a).

The activation of the DSB transcriptional response during seed imbibition significantly demonstrates that even seeds regarded as high quality have accumulated high levels of genotoxic stress not normally encountered in the plant lifecycle. Importantly, this also identifies physiological roles for DSB repair and damage response pathways in plants.

1.7.2 Single strand break repair

A number of pre-genomic era studies established that further to DSBs, there is a correlation between damage to DNA bases and seed quality. In aged rye seeds, loss of viability is associated with an accumulation of SSBs (Cheah and Osborne, 1978b). In solution, DNA is naturally unstable in solution, so orthodox seeds having undergone desiccation often some increased stability to the genome. Work in 2 year old maize kernel demonstrated that base damage to seeds continues to be accumulated during storage when in the dry state but is 6-fold less than the levels of damage observed in solubilised DNA. Upon seed imbibition a 4-fold increase in the number of abasic sites from the dry state is also observed in maize kernels (Dandoy et al., 1987). It is hypothesised that the rapid increase in these sites is due to the function of intermediate DNA damage repair steps in which DNA glycosylases excise damaged bases as part of the BER pathway (section 1.4.3). The up-regulation of genes involved in the repair of oxidative damage upon hydration of an orthodox seed has been observed in

both *Arabidopsis* and *Medicoago truncatula.* This is consistent with a model whereby imbibition is associated with substantial levels of oxidative damage to the genome of the seed (Bailly, 2004).

1.7.2.1 Base excision repair

Among all the DNA lesions induced by ROS, 8-oxoG is particularly common and mutagenic (section 1.3.3). It is a result of ROS-induced hydroxylation of the C-8 position of guanine (Kasai and Nishimura, 1984), which has been shown to significantly increase in seeds during ageing and imbibition (Chen et al., 2012). BER is the major repair pathway to remove 8-oxoG damage (Kimura and Sakaguchi, 2006) and is of potential importance in seed vigour. Work in *Arabidopsis* has demonstrated the over-expression of a BER component, OGG1, enhances seed longevity and abiotic stress tolerance (Chen et al., 2012). *Arabidopsis* seeds deficient in *arp* show increased sensitivity to accelerated ageing (Cordoba-Canero et al., 2014), supporting the importance of BER in maintaining high germination vigour and viability.

1.7.2.2 Nucleotide excision repair

NER is a very versatile DNA repair system composed of two distinct sub-pathways acting on transcriptionally active (Transcription Coupled-Nucleotide Excision Repair (TC-NER)) or inactive DNA respectively (Global Genome-Nucleotide Excision Repair (GG-NER)) (Le May et al., 2010). In *Arabidopsis*, NER is based on damage recognition, incision of the damaged site and release of the oligonucleotide that harbours the lesion (Hays, 2002; Hefner et al., 2003). New nucleotides are then used to synthesise and replace the removed strand of ssDNA (Hays, 2002). The NER associated genes *MtTop1α* and *MtTop1β* in *M. truncatula* are up-regulated in seed (Macovei et al., 2010) suggesting a role of NER in germination.

1.8 Reverse genetics in the study of plant DNA repair

Reverse genetics is the process of studying the phenotypic effect of a mutation in a specific gene as opposed to the classical forward method of identifying mutated genes underlying an already characterised phenotype. Several mutagenized *Arabidopsis* populations have now been generated using *Agrobacteria*-mediated T-DNA insertions (Sussman et al., 2000; Brunaud et al., 2002; Sessions et al., 2002; Alonso et al., 2003; Rosso et al., 2003).

Many thousands of these *Arabidopsis* T-DNA lines are easily identifiable with the use of databases which link the flanking sequence tags of the T-DNA with the position in the genome. Mutant lines are identifiable using the SIGNAL flanking sequence tag database. SIGNAL is the most comprehensive database of T-DNA lines, recording over 360 000 insertion sites covering approximately 90 % of all *Arabidopsis* genes (Li et al., 2007b). The number of genes without T-DNA insertion alleles available may, in part, be explained by the low probability of generating complete coverage of all coding regions given the number of lines available (Alonso et al., 2003). In addition, although T-DNA insertion is generally considered random, with possibly a small amount of micro-homology between the T-DNA border sequence and genomic DNA at the site of incorporation, the integration of T-DNA insertion mutant libraries indicated a preference for T-DNA into A/T-rich regions of chromosomes (Brunaud et al., 2002). Other research has shown that T-DNA shows specificity for the euchromatic regions with proportionately fewer T-DNA insertions in centromeric regions of the genome (Alonso et al., 2003). This apparent specificity may be an artefact of the mutant selection process, whereby the selection of the transformed lines is biased towards those in which expression of the selectable marker is favoured (Kim et al., 2007).

Functional analysis and gene identification using *Arabidopsis* as a model has been greatly accelerated since the advent of such readily available mutant lines and has underpinned it as an important model organism in plant sciences.

1.9 Summary and aims

Despite its importance to agriculture, relatively little is known about the molecular basis of seed quality. A number of pre-genomic era studies established a correlation between seed vigour/longevity and accumulation of genomic damage, but comparatively little work to date has investigated the molecular basis of this.

The objective of this study was to identify molecular components of DNA repair pathways important to seed longevity and vigour. The importance of different repair pathways and their effect on seed vigour and longevity was elucidated through the isolation and analysis of knockout mutants. These studies investigate and compare the roles of four major DNA repair pathways in seed germination and seed quality: NHEJ, HR, BER and NER. Identifying and characterising genes whose action is required following imbibition, will provide a better understanding of the early events central to the germination process.

Studies undertaken to understand the operation of DNA repair pathways in plants have largely been confined to identifying mammalian and yeast homologues. The unique DNA ligase 6, specific to higher plants remains largely uncharacterised but is known to have important roles in seed longevity. Further studies will focus on elucidation of the roles of DNA ligase 6 in planta. Extra-chromosomal recombination assays will provide insights into the roles of DNA ligase 6 in plant recombination activities. Assays to identify PCD events will define the roles of DNA ligase 6 in preferential PCD in root meristem cells in response to genotoxic stress.

An additional aspect of these studies is that most of our understanding of plant DNA repair mechanisms arises from studies in which plants are artificially subjected to irradiation, radiomimetic compounds and other DNA damaging agents. As these genotoxic stresses are not normally encountered in the plant lifecycle, they are not necessarily physiologically relevant. Further elucidation of the roles plant DNA repair pathways in the orthodox seed will provide an increased understanding of their physiological importance.

2. Materials and Methods

2. Materials and methods

2.1 Suppliers

Unless otherwise stated chemicals were obtained from: -

Sigma Aldrich, Poole, UK

2.2 Equipment

Centrifugation of 0.5, 1.5 and 2 ml microcentrifuge tubes used a minispin (Eppendorf) microcentrifuge at 20238 rcf (14680 rpm) at room temperature unless otherwise stated. 15 and 30 ml Falcon tubes (BD Biosciences) were centrifuged using the 5810R centrifuge with the A4-62 rotor. A Beckman Avanti (J-25) refrigerated centrifuge with rotor JLA 16.250 was used for large-scale centrifugation steps in 250 ml tubes (Fisher Scientific).

Polymerase chain reaction (PCR) was performed in the thermocycler machine Mastercycler (Eppendorf). Electrophoresis was performed using a mini subcell electrophoresis tank (BioRad) with a basic BioRad power pack. Solutions and equipment was sterilised using a Radwell Herald autoclave at 121 °C for 20 minutes. Bacterial densities were determined using Biophotometer spectrophotometer (Eppendorf). Nucleic acid concentrations and purity were determined using a ND-1000 spectrophotometer (Nanodrop) with ND-1000 software.

2.3 Plant material

Seed stocks of mutagenized lines of *Arabidopsis thaliana* (hereby referred to as *Arabidopsis*) were obtained from The Nottingham *Arabidopsis* Stock Centre (NASC) (http://*Arabidopsis*.info/) (Scholl et al., 2000) or, if published, from the authors as detailed in table 2.1.

Table 2.1 List of stocks used in this study

Line	*Gene*/ AGI code	Accession Number	Reference/ Stock Centre
Col-0	*N/A*	N1092	NASC
lig4-5	AT5G57160	SALK_095962	(Waterworth et al., 2010)
lig6-1	AT1G66730	SALK_079499	(Waterworth et al., 2010)
ercc1-1	AT3G05210	SALK_033397	(Charbonnel et al., 2010)
ku70-1	AT1G16970	SALK_123114	(Heacock et al., 2004)
ku80-3	AT1G48050	SAIL_714_A04	National *Arabidopsis* stock centre
xrcc2-1	AT5G64520	SALK_029106	(Bleuyard et al., 2005)
arp-1	AT2G41460	SALK_021478	(Córdoba-Cañero et al., 2011)

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

The mutant lines studied were in a background ecotype of Columbia (Col-0) with T-DNA insertion lines obtained from SALK (Alonso et al., 2003), SAIL (Sessions et al., 2002) or GABI (Kleinboelting et al., 2012). PCR based genotyping was used to isolate homozygous single mutants (section 2.7.1) using two week old seedlings grown on half MS media (section 2.4.4), Primer pairs used, were designed to the wild-type allele and T-DNA insertion allele for each gene [Table 2.2]. Isolated homozygous mutants were moved to soil to set seeds (section 2.4.5).

Table 2.2 List of primers

Primer	Sequence
actin7_f	5'-ACATCGTTCTCAGTGGTGGT-3'
actin7_r	5'-GCTGAGGGATGCAAGGATT-3'
arp_tdna	5'-CGAGAAAAAGGCATGAACTTG-3'
arp_wt	5'-GCTGCAGGACCAGAAACTAT-3'
ercc1_tdna	5'-TTTCACCGTATCTTCCTGTGC-3'
ercc1_wt	5'-CAAGTCGTCCGACGTCTACTC-3'
ku70_tdna	5'-CTCTTGGCAAGTACACGCTTC-3
ku70_wt	5'-TTACTTTGTTGTTTCGGGTGC-3'
ku80_f	5'-GATTGTTGCGGGATAAACCA-3'
ku80_r	5'-ATCGCGTTGCTGGAGAATAA -3'
ku80_tdna	5'-AGAGAGTTCAGGGTCCTGCTC-3’
ku80_wt	5'-AGTGGCTGAGGAGAGACTTCC-3'
lig4_tdna	5'-TTTGTTGTTTGAGGATCCGAC-3
lig4_wt	5'-AAAGCCCTAAGGTCTTCATGG-3'
lig6_tdna	5'-GCAAGGATCTTATCCTCCGAG-3
lig6_wt	5'-GTTGGCTCTTCCCCTCCGTGAGAGAC-3'
SAIL_LB1	5’-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3’
SALK_LBb1.3	5'-ATTTTGCCGATTTCGGAAC-3'
xrcc2_tdna	5'-ATCATCATTGGCATTGGAGAC-3
xrcc2_wt	5'-TTTACATCTGGCGATTTTTGC-3

2.3.2 Crossing of *Arabidopsis* to produce double knock-out mutants

Double mutants were produced using two different single T-DNA insertion plants and crossing these (called the F0 generation). For most efficient crossings, plants at a stage when they have developed 5-6 inflorescences and plants that had started to form siliques were chosen. Mature siliques as well as open flowers and buds that have already a white tip were first removed from the first plant as well as open mature buds. Using a pair of Jewelers forceps (5 Inox, Dumont, Switzerland), unopened flower buds were opened by inserting the tip of one pair of forceps between petals and sepals and all immature anthers were removed. From the other plant, mature flowers with visible pollen shedding from the anther were taken intact and the anther was rubbed against the stigma of the mother plant to cover it with pollen grains. Pollinated inflorescences were loosely wrapped in cellophane and red tape to cover and mark the cross. Siliques with hybrid seeds were left to mature for 15-25 days at which point seeds were collected.

2.3.3 Isolation of Double T-DNA insertion knock-out mutants

Hybrid seeds were sown and grown under normal conditions (F1 generation) (section 2.4). These hybrid seeds were heterozygous for the T-DNA insertions inherited from the parental lines. To confirm this, PCR based genotyping was performed (2.7.1) and heterozygous mutants for both genes were allowed to self-fertilise and mature seeds were collected. These seeds were sown and grown under normal conditions to produce the F2 generation of plants, under Mendelian inheritance one in sixteen plants of the F2 generation were homozygous double mutants. To identify homozygous double mutants PCR based genotyping was performed and these were allowed to set seed to ensure a renewable stock.

2.4 Plant Growth Conditions

2.4.1 Seed sterilisation and imbibition

Materials

Sterilisation solution:	10% (v/v) Bleach (5-10% hypocholorite)
	1% (v/v) Triton-X100
Agar media:	0.1% (w/v) Phyto agar (Duchefa Biochemie)
	(Agar media was autoclaved and allowed to cool to room temperature before initial usage).

Method

Arabidopsis seeds were suspended in sterilising solution 1.5 ml sterile microcentrifuge tubes for 10 minutes. The seeds were washed five times in sdH_2O and finally in tissue culture.

2.4.2 Chlorine gas seed surface sterilisation

Materials

Gas sterilisation solution;	100 ml bleach (5-10% hypocholorite)
	3 ml concentrated HCl

Method

Arabidopsis seeds were placed in open microcentrifuge tubes in a sealed desiccator (Nalgene) under a fume cupboard (pf&f Ltd). Chlorine gas was produced by mixing 3ml HCl with 100 ml bleach under the fume cupboard and leaving the solution in the sealed desiccator with the seeds for 3 hours.

2.4.3 Antibiotics

Antibiotic stock solutions and working concentrations are listed in Table 2.3

Table 2.3 Antibiotics used in this study

Antibiotic	Stock Concentration in sdH_2O	Working Concentration
Ampicillin	50 mg ml^{-1}	50 µg ml^{-1}

2.4.4 Plant tissue culture

Materials

Murashige and Skoog media: (pH 5.7)

2.2 g l^{-1} Murashige and Skoog basal medium

1% sucrose

0.8% Phyto agar (for agar plates)

Method

Arabidopsis plants were germinated and grown under sterile tissue culture conditions on a half concentration of Murashige and Skoog media (½ MS) plus 1% sucrose (Murashige and Skoog, 1962). The media was adjusted to pH 5.7 with KOH before adding Phyto agar. Media was autoclaved allowed to cool to 55°C before the appropriate antibiotic/herbicide were added, then poured into 9 cm diameter Petri dishes (Starstedt) and allowed to set. Sterilised *Arabidopsis* seeds (section 2.3) were spread on ½ MS plates under sterile conditions at a typical density of approximately 50 seeds per plate. Plates were sealed with Micropore tape (3M) and the plants grown in a light chamber (MLR-351, http://uk.sanyo.com) unless otherwise stated under a 16 hour photoperiod, 70% humidity at 23 ^{o}C. For growth beyond 2 weeks seedlings were transferred to soil.

2.4.5 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

238 g m^{-3} P_2O_5

408 g m^{-3} K_2O (pH 6.0)

Method

Plants grown beyond 2 weeks were transferred to soil (SHL growing medium). The seedlings were carefully removed from the plates and grown in temperature controlled glasshouses 22 °C, 16 hour photoperiod (long-day conditions).

2.5 Seed production, Seed treatments and Germination assays

2.5.1 Production of seeds

Seeds were produced and given post-after ripening treatments as required in the following manner.

2.5.1.1 High quality and non-dormant seeds

Method

To ensure seeds where of high quality and non-dormant dry mature seeds were harvested from mutant and wild-type plants grown simultaneously as previously described (section 2.4). Seeds from mutant and wild-type plants were produced in the same growth cycle. Seeds were stored in non-airtight 6 ml universal tubes at ambient temperature and humidity. Seeds were left to after-ripened for 2 months prior use to release seeds from dormancy (Finch-Savage and Leubner-Metzger, 2006).

2.5.1.2 Dormant seeds

Method

Plants were grown in tissue culture for 2 weeks and moved to soil, the seedlings were carefully removed from the plates and grown in growth chambers in long day conditions at 15°C. Plants were grown until siliques were mature and harvested. Seeds were not allowed to after ripen by storage at -80 °C until seeds were required.

2.5.2 Germination assays

Method

Seeds were sown on Blue Blotter Germination Paper (SGB1924B, http://www.anchorpaper.com) in 90mm petri dishes (Sarstedt) with 7.5 ml $_dH_2O$ without prior surface sterilisation. Seeds were stratified at 4 °C for 48 hours before being moving them to a growth chamber as previously described (section 2.4.4). Seeds were scored for germination, defined here by emergence

of the radicle from the seed coat (Bewley, 1997), using a stereomicroscope to monitor radicle protrusion. Observations of germination were taken hourly or daily intervals, as appropriate. Independent biological replicates were performed with different harvests of the wild-type and mutant lines for verification of results.

To allow an easy comparison of germination kinetics of each mutant line has been plotted independently with wild-type (Figure 3a-e). The mean time to germination (MTG) is a good measure of seed vigour and is a measure of the average time it will take a seed in a seed lot to germinate upon imbibition (Ranal and Santana, 2006).

2.5.3 Accelerated Ageing of seeds

Method

Seed ageing was performed on high quality non-dormant seeds as previously described for *Arabidopsis* by (Hays, 2002), at a temperature of 40 °C with a relative seed moisture content of 10.8%. Several hundred (~200 mg) seeds of each line were each placed in Eppendorf tubes with the lid removed and suspended over a saturated solution of KCl (50 g KCl in 100 ml H_2O) on an open platform in an air-tight container. The box was placed in a dark incubator at 40 °C for 2 or 4 days. After ageing, the seeds were plated directly on Blue Blotter Germination Paper. The seeds were weighed before and after oven drying at 100 °C for 72 hours to determine moisture loss. The relative moisture content was expressed as a percentage of the fresh weight. Germination assays were then performed (section 2.5.2).

2.5.4 X-ray treatment of seeds and effects on germination and root growth

Method

Seeds were sterilised in 70% ethanol for 5 min, and resuspended in sterile H_2O and stratified at 4 °C for 24 hours. IR treatments of 75 and 130 Gy were delivered using a 320 kV X-ray irradiation system (NDT Equipment Services) at a rate of 1 Gy min^{-1}. Seeds were plated individually on half MS agar plus 1 %

sucrose in 90 mm petri dishes (Sarstadt) grown (section 2.4.4). Plates were then placed upright in a growth chamber and seeds were scored for germination daily (section 2.5.2), after germination has occurred the length of root growth is measured daily to determine effects of root growth.

2.5.5 GA/Fluridone solution for dormancy-breaking treatment of seeds

Dormancy-breaking treatment was used to determine seeds were dormant or non-dormant (Bentsink et al., 2006).

Materials

Citrate/phosphate solution (pH5):

3.3 mM $K_2HPO_4 \cdot 3H_2O$

1.7 mM Citric acid

Gibberellic acid (GA_{4+7}, a mixture of GA4 and GA7 at a ratio of 2:1):

100 µM GA_{4+7} (Duchefa Biochemie)

1 M KOH

50 µM Fluridone (Duchefa Biochemie)

DMSO

Method

To prepare the GA/Fluridone solution, 8.3 mg GA_{4+7} was dissolved in 200 µl 1 M KOH and 4.1 mg Fluridone was dissolved in 1 ml DMSO. These were added to 250 ml Citrate/phosphate solution. Seeds were plated on Blue Blotter Germination Paper with 7.5 ml GA/Fluridone solution and stratified at 4 °C for 48 hours before moving into a growth chamber. Observations of germination were taken at hourly or daily intervals, as appropriate. Controls were performed with H_2O instead of the GA/Fluridone solution.

2.5.6 Cold stress treatment of seeds and effects on germination

Cold-stress treatment was used to determine seed vigour of different seed lots

Method

Seeds were imbibed and incubated at 5 °C for 10 days after which seeds were scored for germination. Seeds were then moved to normal conditions (section 2.5.2) and germination was scored daily thereafter.

2.5.7 Osmopriming treatment of seeds and effects on germination

Materials

PEG 6000 solution

Method

Osmoprimed seeds were prepared by incubating dry mature seeds in the dark, for 5 days at 20 °C, in a −0.75 MPa PEG 6000 solution (2.42 g in 10 ml H_2O). Seeds were then washed in H_2O before being left in the dark on germination paper for 48 hours at ambient temperature to dry out. Primed seeds (n = 25) were then plated, stratified and germinated as previously reported in triplicate.

2.6 Genomic DNA extraction from seed and leaf material

2.6.1 DNA extraction from leaf material for PCR-based genotyping

Material

Extraction buffer:	100 mM Tris-HCl (pH 9.0)
	200 mM LiCl
	50 mM EDTA
	1 % SDS
10 x T.E. buffer:	100 mM Tris-Cl (pH 8.0)
	10 mM EDTA (pH 8.0)

Absolute isopropanol

Method

Between 10 mg – 500 mg leaf material was ground in 1.5 ml microcentrifuge tube containing 500 µl of extraction buffer. The tube was centrifuged (5 minutes, 13 000 rpm) at room temperature after which 350 µl of the supernatant was transferred to a fresh 1.5 ml microcentrifuge containing 350 µl isopropanol. After mixing by inversion, the tube was centrifuged (10 minutes, 13 000 rpm). The supernatant was removed and the pellet was air dried before re-suspension in 100 µl T.E. buffer.

2.6.2 CTAB based Genomic DNA extraction from seed material for gel electrophoresis

Material

CTAB buffer:	2% (w/v) Cetyl trimethylammonium bromide
	2 M LiCl
	20 mM EDTA
	100 mM Tris-HCl pH 9.4
	1 % (w/v) Polyvinylpyrrolidone
	0.2 % (v/v) β-mercaptoethanol
Absolute Isopropanol	
70 % ethanol	
Resuspension buffer:	20 mM EDTA
	100 mM Tris-HCL pH8.0
	250 mM NaCl
T.E. buffer:	10 mM Tris-HCl (pH 8.0)
	1 mM EDTA (pH8.0)
RNase A (10 mg ml^{-1} E.C. 3.127.5)	
Absolute ethanol	

Materials:

Seed material (500 mg) was ground to a fine powder in in liquid nitrogen with a pestle and mortar and placed in a 15 ml falcon tube before 6 ml of preheated (60 ° C) CTAB buffer was added. The tube contents was gently mixed and incubated at 60 ° C for 30 minutes. A chloroform extraction was then performed by added equal volumes of chloroform: isoamyl alcohol (24:1) to the

seed/CTAB buffer mixture followed by centrifugation (3000 rpm, 10 minutes). The aqueous phase (CTAB buffer) was moved into a new falcon tube and the chloroform extraction was repeated. The aqueous phase was recovered and 4.2 ml absolute isopropanol was added to precipitate genomic DNA. The precipitate was recovered by centrifugation (3000 rpm, 5 minutes). The supernatant was removed and the pellet was washed in 10 ml 70 % ethanol. The ethanol was discarded and the pellet air dried (5 minutes). The pellet was resuspended in 650 µl resuspension buffer containing RNase A (10 µg ml^{-1}) and transferred to a 1.5 ml microcentrifuge tube then incubated at 37 °C (30minutes). A chloroform extraction was again performed as before using equal volumes chloroform: isoamyl alcohol (24:1) to Resuspension buffer. The supernatant was transferred to a new microcentrifuge tube and 100 µl absolute ethanol was added drop by drop to a final concentration of 15% to precipitate carbohydrate contaminants. The tubes were incubated on ice for 30 minutes before centrifugation (14 000 rpm, 5 minutes). The supernatant was recovered, 650 µl absolute isopropanol was added to precipitate the DNA and centrifugation was performed (14 000 rpm, 5 minutes) and the supernatant was discarded, retaining the pellet. The pellet was washed in 1 ml 70 % ethanol and spun (14 000 rpm, 1 minute). The ethanol was discarded and the pellet air dried then resuspended in 75 µl T.E. buffer. ND-1000 spectrophotometer (Nanodrop) with ND-1000 software was used to determine the concentration and purity of genomic DNA.

2.7 DNA amplification by Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplification of DNA fragments were conducted using particular DNA polymerase(s) depending on the application. *Thermus aquatcus* Taq DNA polymerase (REDTaq, Thermo Scientific or GoTaq, Promega) was used for all routine DNA amplifications and semi-quantitative DNA amplification.

2.7.1 DNA amplification by PCR

Materials:

ReddyMix PCR Reaction Mix, Thermo Scientific. (final 1 x reaction):

75 mM Tris-HCl (pH 8.8)

20 mM $(NH_4)_2SO_4$

2.0 mM $MgCl_2$

0.01 % (v/v) Tween® 20

dNTP mix (dATP, dCTP, dGTP and dTTP, 0.2 mM each)

0.625 U μl^{-1} ThermoPrime *Taq* DNA polymerase

Precipitant and red dye

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

2 x Green GoTaq® Reaction Buffer (pH 8.5)

3 mM $MgCl_2$.

dNTP mix (dATP, dCTP, dGTP and dTTP, 0.4 mM each)

50 U ml^{-1} *Taq* DNA polymerase

Oligonucleotide primers (Invitrogen and Integrated DNA Technologies,) 10 µM

T.E. buffer (section 2.6)

Method

Oligonucleotide primers were typically designed to have between 20 - 25 bp complementary to the target sequence to be amplified. A 100 µM stock of each oligonucleotide primer was made with T.E. from which a 50 x (10 µM) solution was made in sdH_2O. PCR reactions using REDTaq and GreenTaq pre-mixes used the 1.1 x and 2 x master mixes at a 1 x final concentration. All PCR reaction contained a final concentration of 0.5 µM of forward and reverse oligonucleotide primer and typically 50 – 500 ng templates DNA. PCR reactions were performed in 0.2 ml PCR tubes with the final volume of the reaction mix made up to 20 µl or 50 µl with sdH_2O. Reactions were run in a thermocycler (section 2.2) using the programs described below.

2.7.2 PCR programs

Unless otherwise indicated the following PCR programs were used which depended on the primer pair Tm and PCR product length: Taq50TD

97 °C	1 minute		
97 °C	30 seconds		
55 °C	30 seconds	-0.5 °C/cycle	10 cycles
72 °C	1 minute		
97 °C	30 seconds		
50 °C	30 seconds		35 cycles
72 °C	1 minute		
72 °C	5 minutes		
Taq52TD			
97 °C	1 minute		
97 °C	30 seconds		
57 °C	30 seconds	-0.5 °C/cycle	10 cycles
72 °C	1 minute		
97 °C	30 seconds		
52 °C	30 seconds		35 cycles
72 °C	1 minute		
72 °C	5 minutes		

Taq55TD

97 °C	1 minute		
97 °C	30 seconds		
60 °C	30 seconds	-0.5 °C/cycle	10 cycles
72 °C	1 minute		
97 °C	30 seconds		
66 °C	30 seconds		35 cycles
72 °C	1 minute		
72 °C	5 minutes		

2.8 Gel electrophoresis

2.8.1 Agarose gel electrophoresis

Materials

Tris, Boric Acid, EDTA buffer (T.B.E.) pH8.3:

89 mM Tris base

89 mM Boric acid

2 mM EDTA

Agarose (electrophoresis grade)

Ethidium bromide (10 mg ml^{-1})

Loading buffer: 50 % (v/v) glycerol

0.05 % (w/v) bromophenol blue

0.05 % (w/v) xylene cyanol

Hyperladder I (Bioline)

Methods

Agarose (400 mg) was added to 40 ml T.B.E. and heated in microwave until dissolved. Ethidium bromide (40 x 10 mg ml^{-1}) was added and the gel allowed to set in a gel cast with a comb (BioRad). The gel was overlaid with TBE buffer in the electrophoresis tank (BioRad). Loading buffer (0.25 volumes) was added to the samples which were then loaded on the gel. The marker Hyperladder 1 kb plus (5 µl) was also loaded and run alongside the samples (unless otherwise indicated), which allowed the estimation of the size of the PCR product. Electrophoresis was conducted at 90 V/cm, after which the DNA was visualised on a UV transilluminator and images were analysed using the GeneTools program (Syngene). When percentage gels were required to be lower than 1 %, the initial weight of agarose was altered accordingly.

2.9 Gel extraction

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) *optional*

Methods

The protocol uses a spin column with a silica membrane to purify DNA. The DNA fragment was excised from the agarose gel and weighed in a 1.5 ml microcentrifuge tube. Binding and solubilisation buffer QG was added (300 µl per 100 mg of gel fragment), then incubated at 50 °C until the gel was dissolved and solution turned yellow. If the solution has not turned yellow, 10 µl 3 M sodium acetate was added to the mixture. DNA was precipitated by adding 1 gel volume of absolute isopropanol and mixed. The DNA was bound to a QIAquick spin column silica membrane by centrifugation (1 minute, 12 000 x g). The column was then washed by centrifugation with PE buffer (1 minute, 12 000 x g) twice before the DNA was eluted from the column in 30 µl sdH_2O by centrifugation (2 minutes, 12 000 x g).

2.10 RNA purification and cDNA synthesis

2.10.1 RNA purification

Materials

The SV total RNA isolation kit (Promega) was used for RNA isolation from plant tissue:

SV RNA lysis buffer:	4 M guanidine thiocyanate
	10 mM Tris (pH 7.5)
	0.97 % β-mercaptoethanol
SV RNA wash solution:	60 mM potassium acetate
	10 mM Tris-HCl (pH 7.5)
	60 % ethanol

RNA dilution buffer

3 M LiCl

Absolute ethanol

70 % ethanol

Method

The RNA purification protocol isolates total intact RNA which is purified on a silica spin column. RNA purification was conducted using sterile, autoclaved pipette tips, microcentrifuge tubes, pestle and mortar. The plant tissue was frozen in liquid nitrogen and ground using a freezing cold pestle and mortar. The ground tissue (~100 mg) was added to a 1.5 ml microcentrifuge tubes containing 175 µl RNA lysis buffer and 350 µl RNA dilution buffer and mixed by inversion. The tube was incubated (3 minutes, 70 °C) before centrifugation (14 000 x g, 10 minutes). The lysate was transferred to a fresh 1.5 ml microcentrifuge tube containing 200 µl 95 % ethanol. The mixture was added to a spin column and centrifuged (14 000 g, 1 minute) and the supernatant removed. 600 µl wash buffer was added to the column and re-centrifuged (14

000g , 1 minute), the flow through was removed and a second wash was performed using 250 µl wash buffer (14 000 g, 2 minutes) to remove any residual buffer and the flow through removed again. After changing to a fresh collection tube 50 µl sdH_2O was added to the column and centrifuged (14 000 g, 1 minute) to elute the RNA. The ND-1000 spectrophotometer was used to determine RNA concentration. RNA was then stored at -80 °C.

2.10.2 cDNA synthesis

cDNA synthesis was performed using the SuperScript™ II Reverse Transcriptase Kit (Invitrogen) and total RNA extracted (section 2.10.1);

Materials

200 U μl^{-1} SuperScript™ II Reverse Transcriptase
5 x First-Strand Buffer
0.1M DTT
dNTP mix (dATP, dCTP, dGTP and dTTP, 10 mM each)
500 µg ml^{-1} Oligo-deoxythymidine (Oligo (dT))

Method

To synthesise cDNA the following were added to a 200 µl centrifuge tube; 1 µl Oligo (dT), 500 ng total RNA (section 2.10.1) and 1 µl dNTP mix, with the volume made up to 12 µl with sdH_2O. The tube was incubated (65 °C, 5 minutes) and after chilling on ice the following were added; 4 µl 5 x First-Strand Buffer, 2 µl 0.1 DTT. The mix was then incubated (42°C, 2 minutes) again before 1 µl SuperScript™ II Reverse Transcriptase was added. The mix was then incubated (42 °C, 50 minutes) before inactivation of the reverse transcriptase by a 15 minute incubation at 70 °C. Completed reactions were then stored at –20 °C until required.

2.11 Extra-chromosomal recombination assays

2.11.1 Plasmid preparation for extra-chromosomal recombination

Materials

LB media: 10 g L^{-1} tryptone
5 g L^{-1} yeast extract
10 g L^{-1} NaCl

Ampicillin

Bacto-agar

E. coli

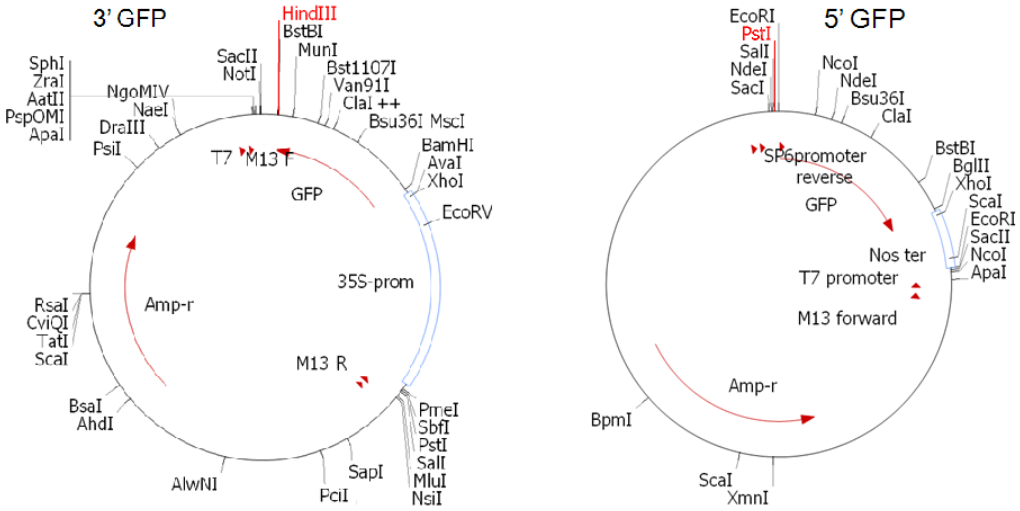
pGEMT Easy vector plasmids

QIAGEN plasmid kit

Methods

E. coli glycerol stocks containing pGEMT Easy vector plasmids (Promega, www.promega.com) including GFP gene inserts with either a 5' or 3' deletion were donated by Dr Christopher West. The plasmid confers ampicillin resistance. Stocks were streaked onto LB media supplemented with 1 % bacto-agar plates supplemented with ampicillin (50 μg ml^{-1}) and incubated at 37 °C for 24 hours. One colony from each plate transferred to 500 ml LB media and incubated overnight (37 °C and 200 rpm shaking). Plasmid was purified using the QIAGEN plasmid isolation kit, and restricted with either HindIII or PstI to obtain line GFP sequences lacking the 3' and 5' respectively [Figure 6-6].

a)



b)

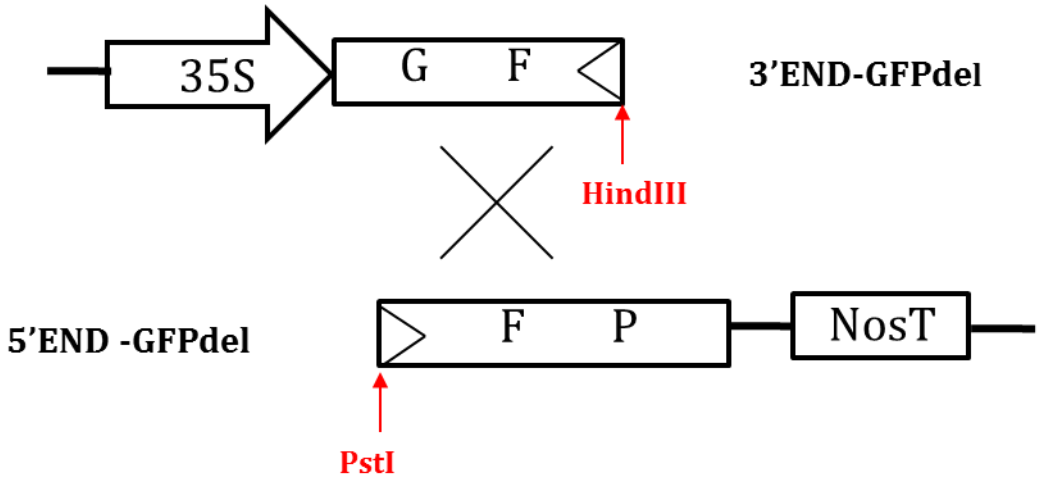


Figure 2-1 Diagram of 3' and 5' GFP delete insertion in pGEMT Easy vector

[A] Schematic of the 3' and 5' GFP delete insertion in pGEMT Easy vector. Digestion sites HindIII and PstI used to linearise the plasmid before protoplast transfection are highlighted **[B]** diagrammatic representation of the 3' and 5' GFP delete insertion and indicating region of homology (587 bp).

2.14.2 Protoplasting, transformation and imaging for extra-chromosomal recombination assay

Materials

Enzyme solution:	4 M mannitol
	10 mM $CaCl_2$
	20 mM KCl,
	20 Mm MES,
	0.1 % cellulose 'Onozuka' R10
	0.25 % Macerozyme
	pH 5.7
W5 solution:	154 mM NaCl
	125 mM $CaCl_2$
	5 mM KCl
	5 mM glucose,
	2 mM MES,
	pH 5.7
MMg solution:	0.4 M mannitol
	15 mM $MgCl_2$
	4 mM MES
	pH 5.7

PEG 4000

Sterile water

Methods

Protoplasts were obtained from 4-5 week old plants as described (Wu et al., 2009). Briefly, freshly cut leaves had the epidermal layer removed by peeling

off previously affixed tape. This leaves mesophyll cells exposed and then were placed in filter-sterilised enzyme solution digesting the cell walls exposed mesophyll cells. The dish was shaken (45 rpm) for 1 hour then centrifuged at 100 rcf for 3 minutes. The pellet is then resuspended in prechilled W5 and protoplasts were then incubated on ice for 30 minutes.

For transfection, protoplasts were centrifuged at 100 rcf for 3 minutes and resuspended in MMg solution. Protoplasts in MMg were mixed with 10µl (2 µg μl^{-1}) of plasmid DNA at room temperature. An equal volume of PEG 4000 solution (40 % (w/v) of PEG 0.1 M $CaCl_2$ and 0.2 M mannitol) was added and incubated for 5 minutes at room temperature. Protoplasts were then gently flooded with W5 solution and centrifuged (100 rcf, 3 minutes). The protoplasts were resuspended in W5 and incubated for 16 hours in dark conditions.

Protoplasts were imaged using an inverted Zeiss LSM 510 META Axiovert 200M confocal microscope (60 x (oil) objective, Argon/2 (458, 477, 488, 514 nm) laser. Protoplasts were illuminated with 543 nm and 488 nm wavelength light to image the red and green fluorescence respectively. Images were analysed with ImageJ by measuring the fluorescence intensity of GFP and dsRED across a section of each transformed protoplast using the ImageJ > Analysis > Analyze Particle... function.

3. Characterisation of the roles NHEJ genes in *Arabidopsis* seed germination

3.1 Introduction

Double strand breaks (DSBs) are the most detrimental form of damage to DNA. In plants, and indeed all eukaryotes, DSBs are repaired by two different DNA repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). NHEJ is the predominant DSB repair mechanism in most higher plant tissues (West et al., 2002; Bleuyard et al., 2005; Waterworth et al., 2011). Whilst NHEJ is a mechanism that rapidly repairs DSBs, it is an error-prone pathway which frequently leads to deletions and/or insertions and therefore has mutagenic consequences. Conversely this is not true of the HR repair mechanism. HR generally facilitates repair without mutation by utilising regions of homology exchanging nucleotide sequences between two similar or identical molecules of DNA, which is then copied to repair the damaged region.

3.1.1 Double strand break repair and DNA ligases

The requirement to seal broken DNA molecules is essential in many biological processes including DNA replication, DNA recombination as well as DNA repair. DNA damage usually results with the production of either a single or double strand break. This may occur either directly as a result of damage and breaking of the sugar-phosphate backbone of DNA or indirectly through the processes of base excision repair and nucleotide excision repair (sections 1.4.3 and 1.4.4). After broken ends have been processed by the SSB or DSB repair machinery, DNA ligases are responsible for re-joining DNA molecules together at the 3' OH and 5' PO_4 ends. The *Arabidopsis* genome contains three DNA ligase genes, *LIG1*, *LIG4* and *LIG6*.

The *LIG4* gene has been well characterised in plants with a well conserved role in the canonical non-homologous ending joining (c-NHEJ) pathway (West et al., 2000). LIG4 joins DNA ends directly in a sequence-independent manner in a pathway involving the KU70-KU80 proteins and the LIG4/XRCC4/XLF complex. LIG4 also interacts with other DNA damage repair factors as suggested by the presence of the BRCA1 C-Terminus (BRCT) domain as previously discussed (section 1.5.3).

The plant-specific *LIG6* was identified by analysis of plant genomic databases (Bonatto et al., 2005). LIG6 displays significant sequence similarity to LIG1 but has a domain structure which is distinct from that of any other DNA ligase. The N-terminus of LIG6 shows high levels of conservation with Pso2/Snm1 proteins. These proteins belong to the Metallo-ß-lactamase associated CPSF ARTEMIS SNM1/PSO2 (ß-CASP) family and serve important roles in the repair of DNA interstrand crosslinks (Bonatto et al., 2005).

3.1.2 Non-homologous end joining repair and the Ku-complex.

NHEJ is a highly conserved pathway DSB repair pathway important in eukaryotes and prokaryotes (Lieber, 2010). The canonical non-homologous end joining pathway is mediated by the KU70–KU80 complex. Seminal work in *S. cerevisiae* and mammalian cell culture demonstrated that KU proteins co-localise within the nucleus and bind exposed dsDNA ends with high affinity as a heterodimer (Dynan and Yoo, 1998; Wang et al., 1998). It was also shown that the KU-complex protects DNA ends from large-scale degradation by exonucleases including Exo1 in *S. cerevisiae* (Mimitou and Symington, 2010). In eukaryotes, the KU-complex is additionally involved in maintaining the stability of telomeres. Exo1 resects Ku-depleted blunt-ended telomeres and promotes homologous recombination (Kazda et al., 2012).

Arabidopsis KU70 and *KU80* have a constitutive low-level expression in all tissues. After exposure to DNA-damaging agents, a more than threefold induction of *KU70* and *KU80* expression is observed in protoplasts (Tamura et al., 2002). *In vitro* work has demonstrated their interaction both with each other and DNA ends, in addition *ku80*-deficient mutant lines display hypersensitivity to the DNA damaging agents menadione, bleomycin and γ-irradiation (West et al., 2002).

3.1.3 Redundancy in NHEJ pathways

Recent studies in mammalian systems have also elucidated the high occurrence of cross-talk between repair pathways (Nussenzweig and Nussenzweig, 2007) and this work has begun to be transferred to our understanding in *Arabidopsis*. (Charbonnel et al., 2011). For example, kinetic analysis of DNA DSB repair pathways in *Arabidopsis* has shown that when *KU80* is knocked out and the classically defined canonical-NHEJ (c-NHEJ) (section 1.5.3.1) is suppressed, SSB machinery is then utilised in a back-up form of NHEJ occurs to repair the DSB (Charbonnel et al., 2010; Charbonnel et al., 2011). *Arabidopsis ku70* and *ku80* mutants display a greater dependence on the use of micro-homologies between the DNA ends and a lower frequency of insertions of filler DNA at the break site (Heacock et al., 2004).

3.1.4 Analysis of NHEJ and double strand break repair associated genes in *Arabidopsis* seeds

Gamma irradiation causes an array of cellular damage, including DSBs, and leads to slowed seed germination in *Arabidopsis.* When irradiated, the NHEJ mutants *lig4* and *ku80* display an increased delay to germination completion when compared to wild-type (Friesner and Britt, 2003). In addition, seeds deficient in *ku70* exhibit a hypersensitivity to the alkylating agent methyl methanesulfonate (MMS) (Riha et al., 2002). Excision of damaged bases has the potential to generate DSBs through the conversion of SSBs into DSBs, typically during DNA replication. MMS hypersensitivity in *ku70*-deficient seeds was only observed at the seed stage of the plant lifecycle (Riha et al., 2002) indicating a higher requirement for KU70-dependent DNA repair specifically during germination.

DNA ligases are the enzymes responsible for the final DNA end-joining step in almost all DNA repair pathways. *LIG4* in *Arabidopsis* is well characterised as the ligase involved in the canonical NHEJ pathway. Another, less well characterised DNA ligase, the plant specific *LIG6,* may also be involved in DSB repair as mutants display a reduced accuracy of the DSB repair pathway (Huefner et al.,

2011). Mutant *lig6* plants also display hypersensitivity to X-rays, although the reduction in growth is not as severe as that observed in *lig4* mutant lines. Mutants in both *LIG4* and *LIG6* display a hypersensitivity to accelerated ageing, displaying decreased vigour and viability (Waterworth et al., 2010). This established a genetic link between DSBs and seed longevity. A chromosome region containing *LIG4* in *Arabidopsis* was subsequently identified in a QTL study for seed longevity (Nguyen et al., 2012) potentially providing additional support for the DSB repair machinery in maintaining high seed quality.

3.1.5 Aims

Most work demonstrating the roles of DSB repair machinery in plant biology has been conducted using non-physically relevant treatments including X-rays, and radiomimetic agents such as bleomycin. Recent work has begun to focus more on physiologically relevant analyses such as accelerated ageing demonstrated in the analyses of *LIG4* and *LIG6* function in germination (Waterworth et al., 2010).

Through identifying and analysing knock-out mutants of different c-NHEJ components, the work reported in this chapter further validates previous work and further extends the premise that the DSBR machinery in *Arabidopsis* is required for seed viability, vigour and seed longevity.

3.2 Results

3.2.1 Double strand break repair in *lig6-1* and *lig4-5* and double mutant lines after seed priming

3.2.1.1 Isolation of *lig6-1* and *lig4-5* and double mutant lines

To further advance the understanding of the role of *LIG6* in DNA repair, a knock-out mutant line for *LIG6* (AT1G66730) was isolated (SALK_079499) along with a *LIG4* (AT5G57160) mutant (SALK_079499) and the double *lig6-1 lig4-5* mutant [Figure 3-1].

Both mutant T-DNA insertions are originally from the SALK T-DNA mutant collection and are the result of the *Agrobacterium tumefaciens*-mediated integration of the T-DNA region of the pROK2 vector (Alonso et al., 2003). The rationale for analysing mutant lines for both DNA ligases was to allow comparisons along with identification of independent or redundant repair functions of both genes.

These T-DNA lines have previously been isolated and characterised (Waterworth et al., 2010). The T-DNA insertions in these cases have been shown to disrupt the expression of the *LIG6* and *LIG4* transcripts. To ensure the presence of homozygous T-DNA insertion alleles, PCR analysis was conducted utilising primers corresponding to the LB regions of the T-DNA inserts and gene specific primers as shown in [Figure 3-1]. The wild-type allele was detected using primer pairs designed to the *LIG6* and *LIG4* genomic sequences. As these primers spanned the region of T-DNA insertion and due to the large size of the T-DNA (roughly 4 500 bp) (Alonso et al., 2003) along with PCR cycle parameters (section 2.7.2: Taq55TD), a PCR product that contained the entire T-DNA insertion was unable to be amplified, therefore presence of the T-DNA results in a failure to amplify the wild type gene.

PCR analysis shows that all three mutant lines have homozygous alleles containing their respective T-DNA insertions, with only bands present for the T-DNA insertions in the mutant lines [Figure 3-2]. Controls show the wild type gene in Col-0 lines.

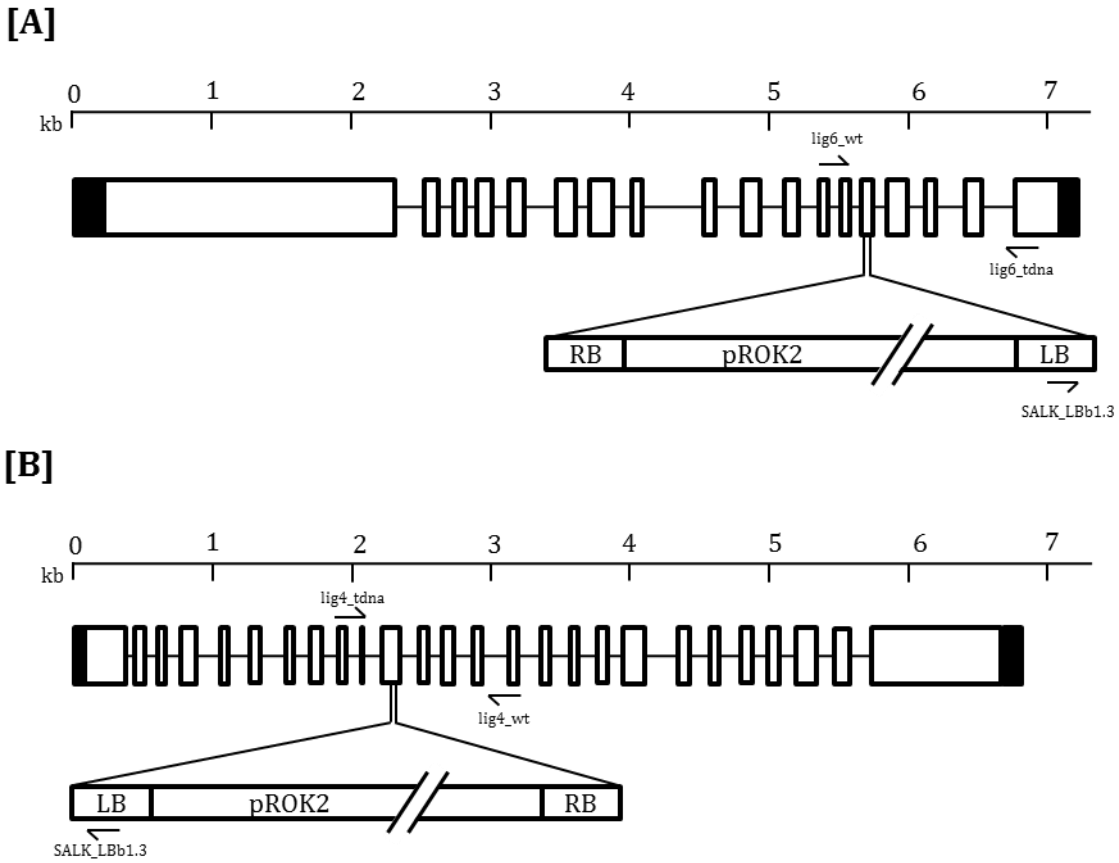


Figure 3-1 The *LIG4* and *LIG6* genes and position of T-DNA insertion

Exons are denoted by boxes, with filled boxes representing UTR, empty boxes representing the coded regions and introns represented by a line.

[A] Schematic of the *Arabidopsis LIG6* gene. The position of the T-DNA insertion is indicated in the 14th exon. The location of the primers used for genotyping *lig6-1* are shown, with the T-DNA allele identified using primers lig6_tdna and SALK_LBb1.3 and the wild-type allele identified using primers lig6_tdna and lig6_wt [table 2.2].

[B] Schematic of the *Arabidopsis LIG4* gene. The position of the T-DNA insertion is indicated in the 11th exon. The location of the primers used for genotyping *lig4-5* is shown, with the T-DNA allele identified using primers lig4_tdna and SALK_LBb1.3 and the wild-type allele identified using lig4_tdna and lig4_wt primers [Table 2.2].

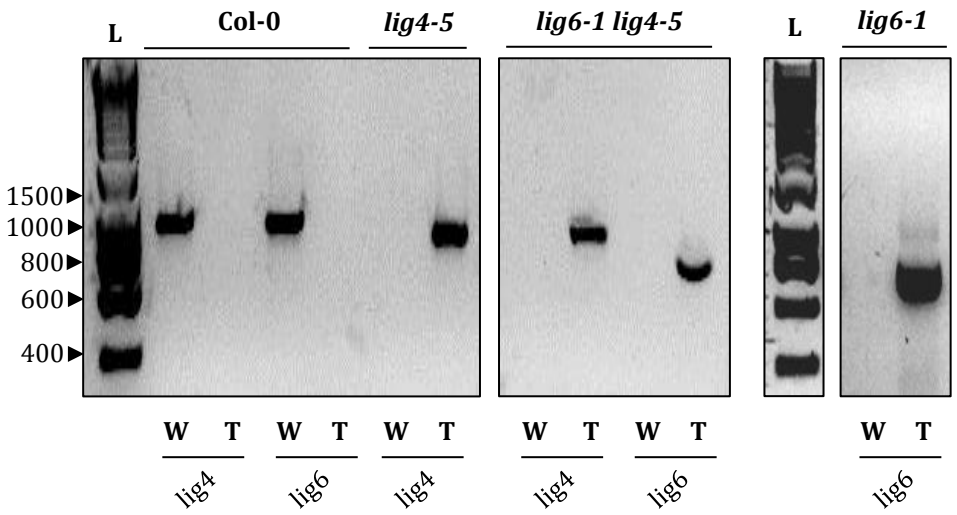


Figure 3-2 Isolation of *lig6-1* and *lig4-5* T-DNA insertion mutant.

Left Border PCR analysis of the*lig6-1* and *lig4-5* T-DNA insertion lines. Genotyping to identify wild-type (WT) and homozygous plants for the T-DNA alleles. The PCR used primers shown in Figure 4-1 [A]. The ladder used was Hyperladder 1 kb plus (lanes L). Wild-type plants resulted in only a wild-type band (lane W) and homozygous plants had only the T-DNA band (lane T). PCR amplification of genomic DNA (section 2.7) was performed using DreamTaq PCR reaction mastermix (section 2.7.1) with the PCR program Taq55TD (section 2.7.2) in 20 µl.

3.2.1.2 *lig6-1, lig4-5* and double mutant lines exhibit decreased seed longevity and germination vigour

Previous work has shown the requirement for *LIG6* and *LIG4* in high seed germination vigour. Seeds lacking either DNA ligase take a longer time to germinate when compared to wild-type and so show an increased mean germination time (MGT) (Waterworth et al., 2010). Further to this, null mutant seeds are hyper-sensitive to accelerated ageing treatment (section 1.1.4.1). Mutant lines display increased MGT and decreased viability (Waterworth et al., 2010) demonstrating a role for both ligases in seed longevity.

To test for vigour differences, the MGT (hours) was plotted for each genotype. MGT was measured by adding together the time taken for each seed that did germinate together and dividing that value by the number of seeds that germinated. This can be expressed in the following equation [equation 3-1]:

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

Equation 3-1 Mean germination time equation

t_i is time starting of the experiment (after stratification) to the i^{th} observed (hour); n_i is the number of seeds germinated in the i^{th} time interval, and k is the point at which the last seed that will germinate does so. The mean germination time of each independent test was the averaged to give the final MGT value.

To confirm the previously reported phenotypes of reduced seed vigour and hypersensitivity to accelerated ageing of *lig6* and *lig4* (Waterworth et al., 2010), germination assays were performed. Forty seeds of each genotype were plated in triplicate onto germination paper (Anchor Blue, California) in a 90 mm petri dish with 7.5 ml dH_2O and stratified at 4 °C for 2 days before being moved to a growth chamber (section 2.5). Germination was scored at time points up to 80

hours and subsequently converted into a percentage of seeds germinated from total lot relative to wild type controls. Germination is scored as protrusion of the radicle from the seed coat, the well-established industry standard way of defining germination (Ellis and Roberts, 1978).

To quantify whether there was a significant difference between the means of each genotype, a one-way analysis of variance (ANOVA) was used and relevant post hoc test (see below) implemented to determine differences between individual groups. As in the previous chapters, groups that met all assumptions of equal sample number and equal variance between the same groups used the Tukey HSD test. Groups which failed to meet either one or both assumptions used the Tukey HSD test with the Games-Howell correction to account for these violations. To test whether sample groups had equal variance the Levene's Test for Equality of Variances was applied. $P \leq 0.05$ indicates samples groups do not display equal variance.

Germination of high quality (section 2.5) wild-type, *lig6-1*, *lig4-5* and *lig6-1 lig4-5* seed lots all resulted in c. 100% viability [Figure 3-3 A and B]. Post hoc analysis confirms there was significant difference between wild-type MGT and that of the mutant lines; *lig6-1* (P = 0.04), lig4-5 (P = 0.006) and *lig6-1 lig4-5* (P = 0.006) [Figure 3-3 C]. This data supports the previous work in these mutant lines (Waterworth et al., 2010). Further germination analysis of 2 days aged mutant lines showed a significant decrease in the viability of each mutant line when compare to wild-type; *lig6-1* (P = 0.039), *lig4-5* (P = 0.047) and *lig6-1 lig4-5* (P = 0.002) and a significant increase in MGT; *lig6-1* (P = 0.000), *lig4-5* (P = 0.006) and *lig6-1 lig4-5* (P = 0.000). This also concurs with the previously reported data (Waterworth et al., 2010) .

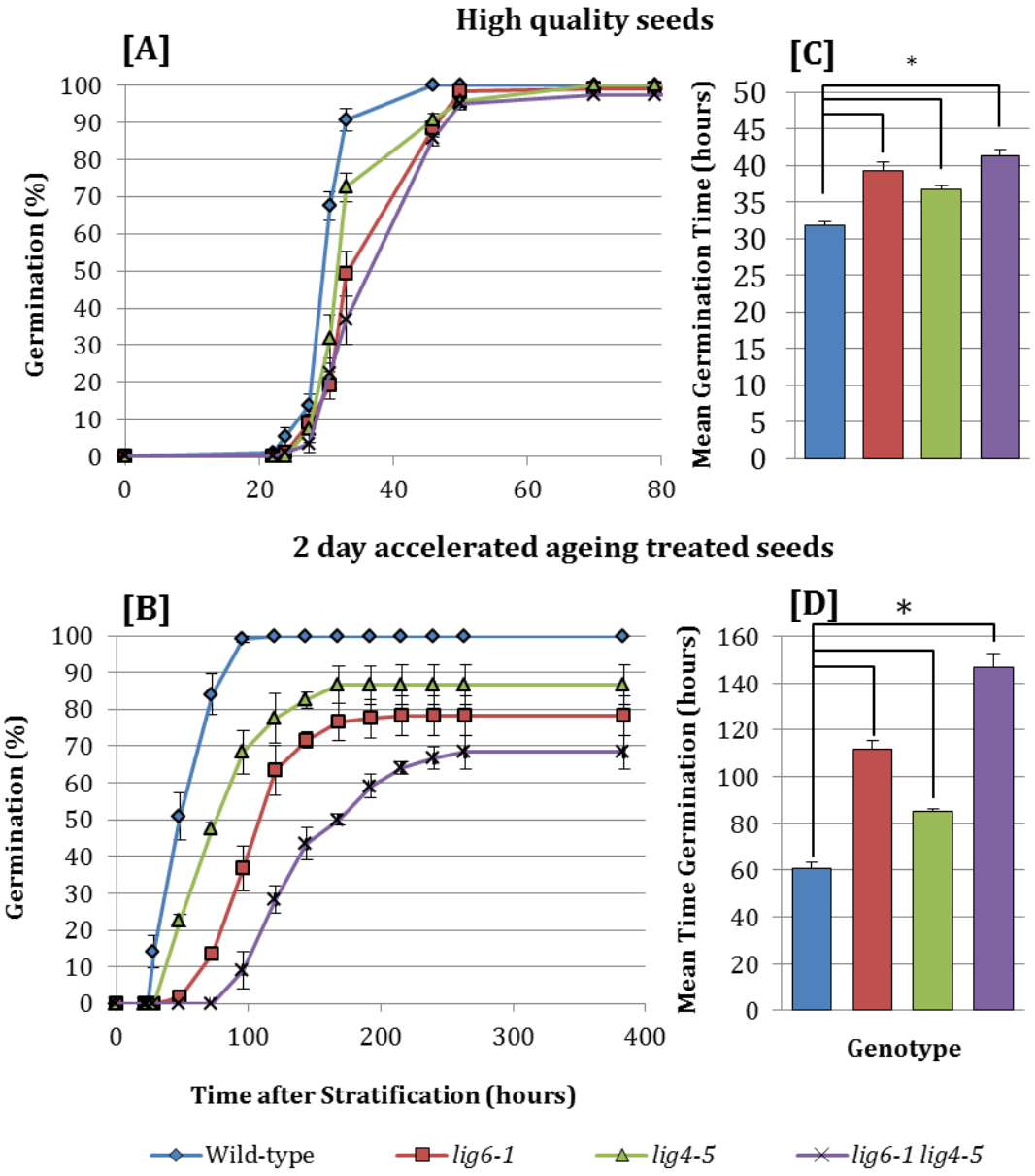


Figure 3-3 Germination performance of *lig6-1*, *lig4-5* and the double knock-out mutant in high quality seeds.

[A and C] Comparison of wild-type and mutant *lig6-1*, *lig4-5* and *lig6-1 lig4-5* seeds germination. Germination performance was analysed at 23 °C under 16 hour light and 8 hour dark cycle conditions. Seeds (n=40) were plated in triplicate on to germination paper and stratified at 4 °C for 48 hours before transfer to 23 °C (section 2.4). Error bars show the standard error of the mean (SEM). **[A] Unaged seeds germination performance,** Total viability for; Wild-type M = 100.00 % (SEM = 0), *lig6-1* M = 99.17 (SEM = 1.44), *lig4-5* M = 100 (SEM = 0) and *lig6-1 lig4-5* M = 98.33 (SEM = 1.13). The probability associated with Levene's Test showed equal variance (p=0.004). Post hoc comparisons using the Tukey HSD test indicated final viability are not significant between any of the genotypes (P > 0.05). **[C] MGT of unaged seeds,** Mean germination time (MGT) for physiological germination is presented. Wild-type M = 31.91 hours (SEM = 0.48), *lig6-1* M = 39.35 hours (SEM = 1.18), *lig4-5* M = 36.76 (SEM

= 0.44) and *lig6-1 lig4-5* M = 41.27 (SEM = 0.85). Levene's Test did show equal variance (p=0.321) Using the Tukey HSD test with the Games-Howell correction all three mutant lines were shown to be significantly different to wild-type; *lig6-1* (P = 0.04), *lig4-5* (P = 0.006) and *lig6-1 lig4-5* (P = 0.006).

[B and D] Germination of 2 days accelerated ageing seeds at 40 °C over a saturated KCl solution at 82 % relative humidity. Error bars represent the standard error of the mean. Values are from three replicates of 40 seeds each. Germination was analysed and displayed as in **[A and C]. Graph [B]** shows total viability for; Wild-type M = 100.00 % (SEM = 0), *lig6-1* M = 78.33 (SEM = 5.46), *lig4-5* M = 86.67 (SEM = 1.67) and *lig6-1 lig4-5* M = 64.17 (SEM = 4.39). The probability associated with Levene's Test did show equal variance (P = 0.03). Post hoc comparisons using the Tukey HSD indicated significant difference between wild-type and all three mutant lines; *lig6-1* (P = 0.039), *lig4-5* (P = 0.047) and *lig6-1 lig4-5* (P = 0.002). **[D] MGT of 2 days aged seeds** Mean germination time (MGT) for physiological germination is presented. Wild-type M = 61.00 hours (SEM = 2.68), *lig6-1* M = 111.59 hours (SEM = 1.60), *lig4-5* M =83.44 hours (SEM =2.17) and *lig6-1 lig4-5* M = 146.86 (SEM = 5.55). Levene's Test did not show equal variance (P = 0.049) Post hoc comparisons using the Tukey HSD indicated significant difference between wild-type and all three mutant lines; *lig6-1* (P = 0.000), *lig4-5* (P = 0.006) and *lig6-1 lig4-5* (P = 0.000).

This data, along with supporting experiments which included germination under cold stress conditions (Waterworth et al., 2010), led to the hypothesis that the delayed germination observed in these mutants may result from a reduced DNA repair capacity. Therefore this work supports the repair hypothesis specifically the need for the seed to repair damage accrued before imbibition (section 1.1.4.1). In seeds with poor vigour, which are thought to have increased damage and reduced repair capacity (Matthews et al., 2012), pre-sowing germination treatments can be performed on the seed lot which can restore good seed vigour. One such treatment is known as osmopriming and is widely used commercially in the agronomical world to ensure high vigour seeds at the point of sowing (section 1.1.4.3).

The molecular mechanisms which underlie the improvement of vigour by priming remain unclear but are thought to involve repair processes and advanced germination progression. A major problem associated with priming is that seed longevity is reduced; however, the reasons for this remain unclear. This raises the question as to whether such priming treatments can promote the repair of DSBs by using back-up repair pathways (section 1.5.3.2) in these mutant lines whereby reduced DSB repair capacity caused lower vigour and loss of viability.

3.2.1.3 Osmopriming of *lig6-1* and *lig4-5* and double mutant line seeds eliminates the delay in seed germination in high quality seeds

To examine if priming treatments can change the mutant seed germination performance relative to wild-type, seeds underwent osmopriming treatment before germination analysis was performed. The osmopriming protocol used was adapted from a previously published protocol (section 2.5.7) (Gallardo et al., 2001). Osmoprimed seeds were prepared by incubating dry mature seeds in the dark, for 5 days at 20 °C, in a −0.75 MPa PEG 6000 solution (Michel and Kaufmann, 1973). Seeds were then washed in dH_2O before being left in the dark on germination paper for 48 hours at ambient temperature. Primed seeds (n =

25) were then plated, stratified and germinated as previously reported in triplicate (section 2.5.2). Wild-type seeds (n = 30) that had been treated with a course of 5 days accelerated ageing (section 2.5.3) were compared to non-primed, aged seeds [Figure 3-4]

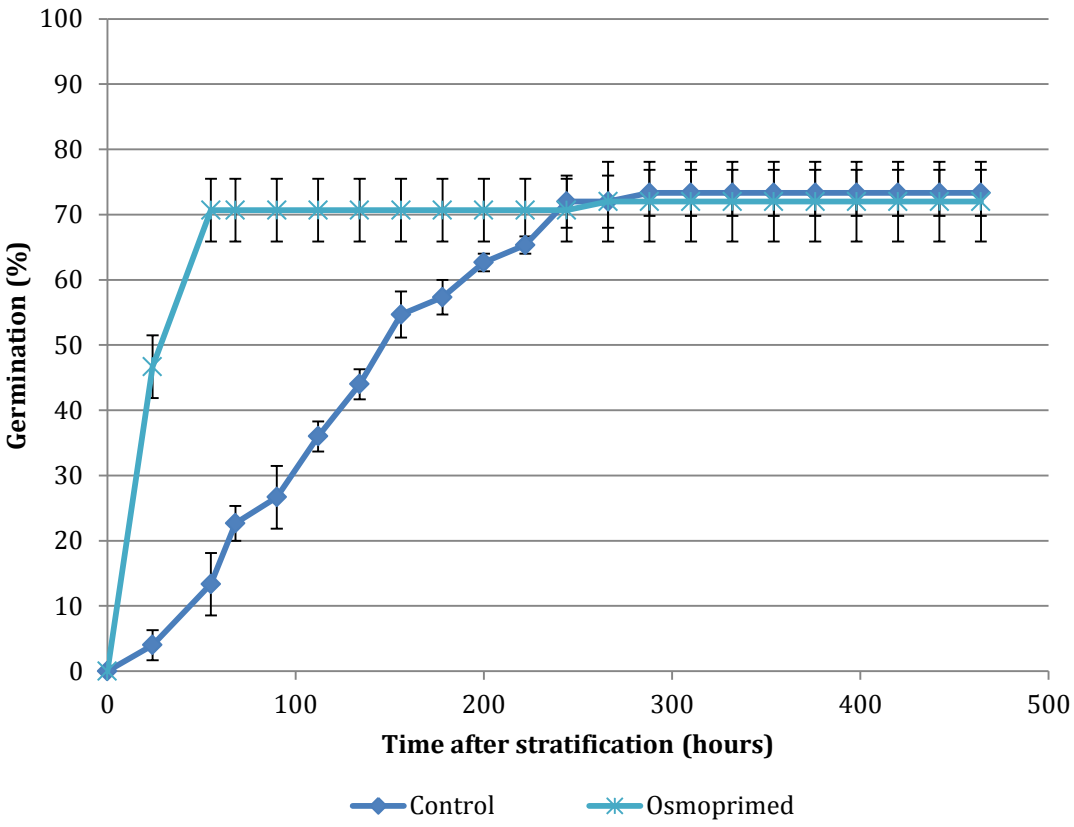


Figure 3-4 Germination performance of 5 days accelerated aged wild-type seeds with and without priming

Germination performance was analysed at 23 °C under 16 hour light and 8 hour dark cycle conditions. 5 day aged wild-type seeds (40 °C, 82 % relative humidity) had had undergone 5 days of osmopriming were plated (n =25) in triplicate on germination paper and stratified at 4 °C for 48 hours before transfer to 23 °C long-day conditions (section 2.5). 5 day aged wild-type seeds that had not undergone any priming treatment were used as a control group. Primed seeds had a total viability = 73.33 % (SEM = 3.52) and MGT = 38.05 hours (SEM = 6.95) and control seeds had a total viability = 72.00 % (SEM = 6.11) and a MGT = 138.46 hours (SEM = 8.77). Student T-Test analysis indicates there is no significant difference between primed and control seed viability (P = 0.86). A statistically significant difference is observed between primed and control seed MGT (P = 0.0009).

The students two-tailed T-test was preformed to analyse the MGT of the primed seeds (38.05 hours) compared to the control seed group (138.46 hours). As there are only two groups to compare, a one-way ANOVA would be inappropriate. Analysis showed that priming wild-type *Arabidopsis* seeds improves vigour aged seeds that have been primed germinate significantly faster (P = 0.0009) than aged seeds that have not been osmoprimed and therefore demonstrates successful application of this technique.

Osmopriming was then performed on the *lig6-1*, *lig4-5* and *lig6-1 lig4-5* mutant seed lines including a wild-type control group. Two separate experiments were performed; one osmopriming high quality seeds [Figure 3-5 A and C] and the other osmopriming two day aged seeds [Figure 3-5 B and D].

Mutant seeds deficient in *LIG6, LIG4* or both germinate with lower vigour than wild-type after accelerated ageing [Figure 3-5 A and C] (Waterworth et al., 2010). However, after priming any significant delay in MGT was lost in all mutant backgrounds with respect to wild-type (*lig6-1* P = 0.556, *lig4-5* P = 0.802 and *lig6-1 lig4-5* P = 0.136; using a one-way ANOVA with Tukey HSD post hoc analysis). This suggested that repair processes are operative during priming presumably through redundant pathways. It was most probably that *LIG1* as the only remaining ligase in the *lig6-1 lig4-5* line is sufficient to overcome the observed reduced repair capacity (Waterworth et al., 2010). Furthermore there was no detrimental effect to total viability after priming in unaged seeds when comparing the different genotypes (P > 0.05).

After 2 days ageing with subsequent osmopriming [Figure 3-5 B] differences were observed however. All mutant lines displayed improved vigour but lower viability compared to wild-type; *lig6-1* (P = 0.01), *lig4-5* (P = 0.04) and *lig6-1 lig4-5* (P = 0.000). This indicated osmopriming as performed here on aged mutant lines (section 2.5.7) had deleterious effects on viability. This finding was further emphasised when the MGT of these lines was analysed [Figure 5-6 D]. The *lig6-1* and *lig6-1 lig4-5* lines had significantly higher MGTs (P≤0.002) than wild-type. Interestingly, the MGT of *lig4-5* was not significantly different from wild-type but was significantly shorter than *lig6-1* (P = 0.027) and *lig6-1 lig4-5*

(P = 0.002). This suggested that *LIG6* could have a specific role in seed germination that cannot be fully compensated for by either LIG1 or LIG4.

This work built on the previously characterised roles of *LIG4* and *LIG6* in seed germination by raising the question: was this decrease in vigour specific to DNA ligases or do other DSB repair factors also influence seed vigour and viability? Whilst it is thought *LIG6* has a role in repairing DSBs, the specific pathway it operates through remains elusive (Waterworth et al., 2010). *LIG4* on the other hand is a well characterised gene in *Arabidopsis* with highly conserved function in the eukaryotic kingdom, critical to the NHEJ pathway (West et al., 2000). DNA repair pathways are multistep mechanisms and it was therefore decided to examine different components of the NHEJ pathway to determine whether the NHEJ pathway was more widely involved in seed vigour.

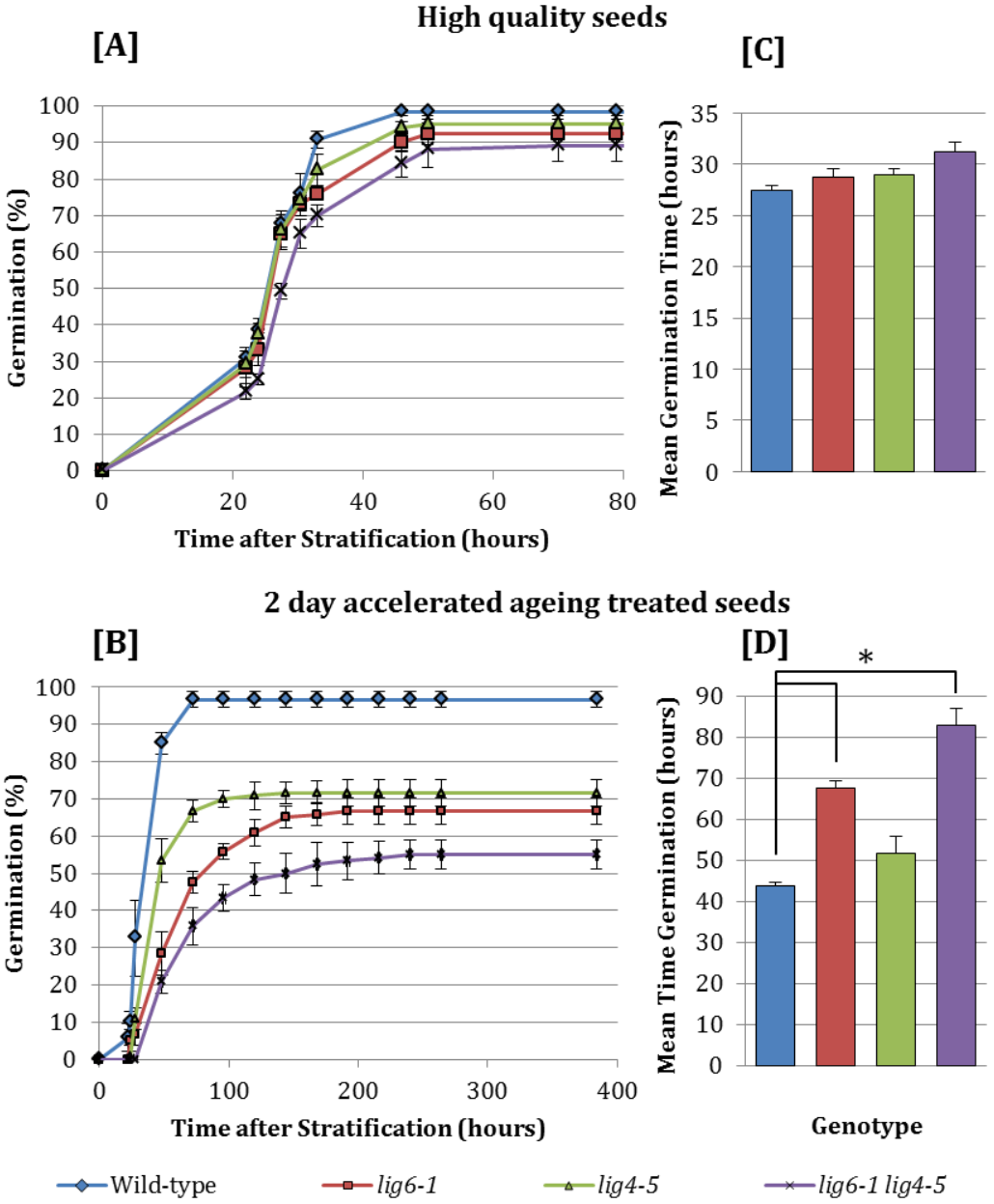


Figure 3-5 Germination performance of DNA ligase mutant seeds after osmopriming

[A and C] Comparison of high quality wild-type and mutant *lig6-1*, *lig4-5* and *lig6-1 lig4-5* seeds after 5 days osmopriming treatment. Germination performance was analysed as previously described (Section 2.5). (n=40), error bars show SEM. **[A] High quality seeds germination performance.** Total viability for: Wild-type M = 98.33 % (SEM = 0.83), *lig6-1* M = 93.33% (SEM = 2.20), *lig4-5* M = 95.0 % (SEM = 2.50) and *lig6-1 lig4-5* M = 89.12% (SEM = 1.49). The probability associated with Levene's Test showed equal variance (p=0.116). Post hoc comparisons using the Tukey HSD test indicated final viability not to be significant between any of the genotypes (P > 0.05). **[C] MGT of High quality seeds,** Mean germination time (MGT) for physiological germination is presented. Wild-type M = 27.49 hours (SEM = 0.46), *lig6-1* M = 29.04 hours (SEM = 0.85), *lig4-5* M = 28.71 hours (SEM = 0.89) and *lig6-1 lig4-5* M = 41.27 hours (SEM = 1.02). Levene's Test show equal variance (p=0.28). Using the Tukey HSD test all three mutant lines were shown to not be significantly different to wild-type; (P > 0.05).

[B and D] Germination of DNA ligase mutant lines after 2 days accelerated ageing. Germination assays performed and analysed as above. **Graph [B]** shows germination performance and total viability for: Wild-type M = 96.67% (SEM = 2.20), *lig6-1* M = 63.33% (SEM = 1.67), *lig4-5* M = 71.67% (SEM = 4.41) and *lig6-1 lig4-5* M = 54.17% (SEM = 4.41). The probability associated with Levene's Test did show equal variance (p=0.324). Post hoc comparisons using the Tukey HSD indicated significant difference between wild-type and all three mutant lines; *lig6-1* (P = 0.01), *lig4-5* (P = 0.04) and *lig6-1 lig4-5* (P = 0.000). **[D] MGT of the 2 days aged seeds:** Mean germination time (MGT) for physiological germination is presented. Wild-type M = 43.68 hours (SEM = 1.83), *lig6-1* M = 67.49 hours (SEM = 4.07), *lig4-5* M = 51.79 hours (SEM = 1.68) and *lig6-1 lig4-5* M = 82.84(SEM = 4.05). Levene's Test did show equal variance (P = 0.140) Post hoc comparisons using the Tukey HSD indicated significant difference between wild-type and the *lig6-1* and double *lig6-1 lig4-5* mutant lines; *lig6-1* (P = 0.002), and *lig6-1 lig4-5* (P = 0.000).

3.2.2 The roles of *KU70* and *KU80* in seed quality

3.2.2.1 Isolation of *ku70* and *ku80*-deficient lines

To investigate further potential roles for NHEJ factors in maintenance of seed viability, vigour and longevity, the core NHEJ components KU70 and KU80 were selected for analysis. The choice of genes for investigation in this study were done so using the following criteria: a clear understanding of the roles the gene has in this repair pathway and well characterised hypersensitivity to DNA damage in knock-out mutants lines. This hypersensitivity demonstrates the genes do not display redundancy in that repair pathway.

KU70 and KU80 form a dimer which binds broken DNA ends stabilising and protecting the exposed DNA. The complex then recruits other NHEJ components to facilitate end-joining and ligation (section 1.5.3). The KU genes have been previously well characterised in *Arabidopsis* and their roles in NHEJ are well understood (section 3.1.2). T-DNA insertion lines were subsequently isolated in *KU70* (AT1G16970) and *KU80* (AT1G48050).

A previously characterised T-DNA insertion for *KU70* (*ku70-1*) (Heacock et al., 2004) was identified using the SIGNAL flanking sequence tag database (http://signal.salk.edu/cgi-bin/tdnaexpress) and ordered through the Nottingham *Arabidopsis* Stock Centre (NASC, http://*Arabidopsis*.info) (Scholl et al., 2000) (section 2.3). The *ku70* mutant line was obtained from the SALK T-DNA mutant collection (Alonso et al., 2003). A previously uncharacterised *ku80* mutant line was selected from the SAIL T-DNA mutant collection (Sessions et al., 2002). Primers for the mutant lines were designated for *ku70-1* (SALK_123114) [Figure 3-6] and *ku80-3* (SAIL_714_A04) [Figure 3-7]. The *ku70-1* line was produced as a result of *Agrobacterium tumefaciens*-mediated integration of the T-DNA region of the pROK2 vector and the *ku80-3* line are have an integration of the T-DNA region of the pCSA110 vector.

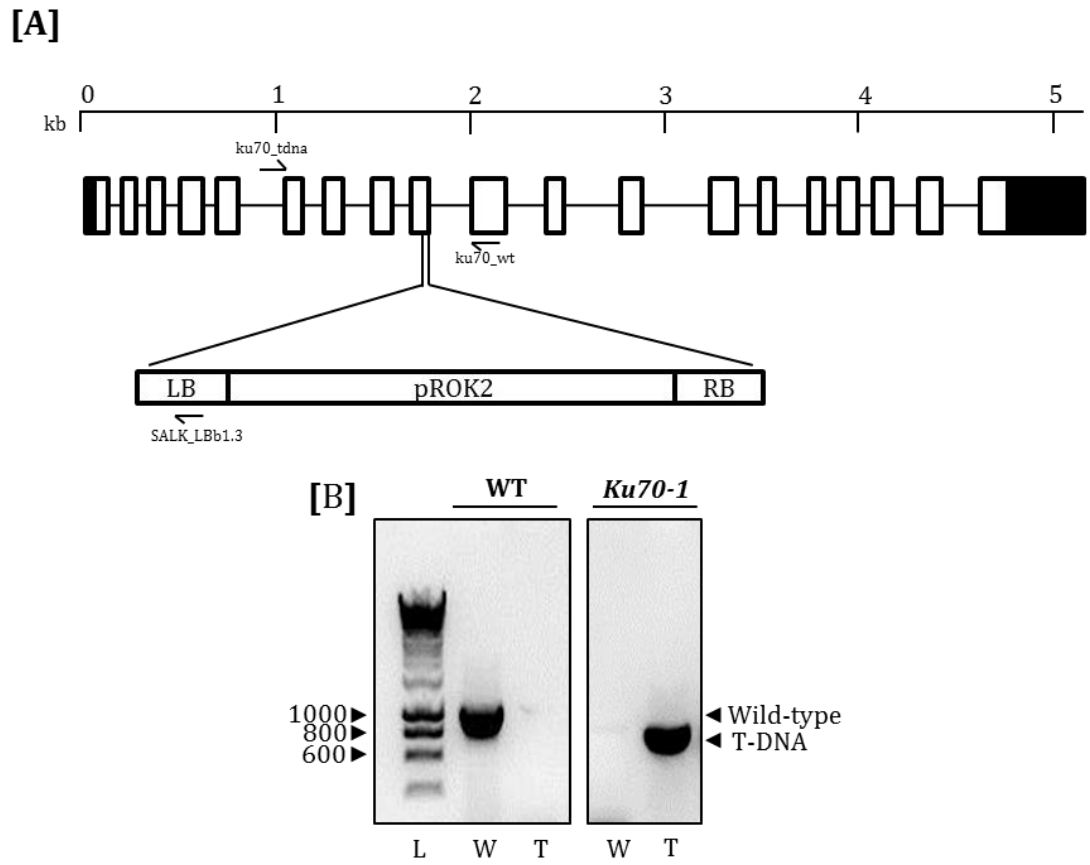


Figure 3-6 Isolation of *ku70-1* T-DNA insertion mutant.

[A] Schematic of the *Arabidopsis KU70* gene. Exons are denoted by boxes, with filled boxes representing UTR, empty boxes representing the coded regions and introns represented by a line. The position of the T-DNA insertion is indicated in the 9th exon as previously described (Heacock et al., 2004). The location of the primers used for genotyping *ku70-1* are shown, with the T-DNA allele identified using primers ku70_tdna and SALK_LBb1.3 and the wild-type allele identified using primers ku70_tdna and ku70_wt [Table2-2]

[B] Left Border PCR of *ku70-1* T-DNA insertion. PCR based genotyping to identify wild-type (WT) and homozygous (*ku70-1)* T-DNA insertion plants. The PCR used primers shown in Figure 4-1 [A]. The ladder used was Hyperladder 1 kb plus (lane L). Wild-type plants resulted in only a wild-type band (lane W) and homozygous plants had only the T-DNA band (lane T). PCR amplification of genomic DNA (section 2.7) was performed using DreamTaq PCR reaction mastermix (section 2.7.1) with the PCR program Taq52TD (section 2.7.2) in 20 µl.

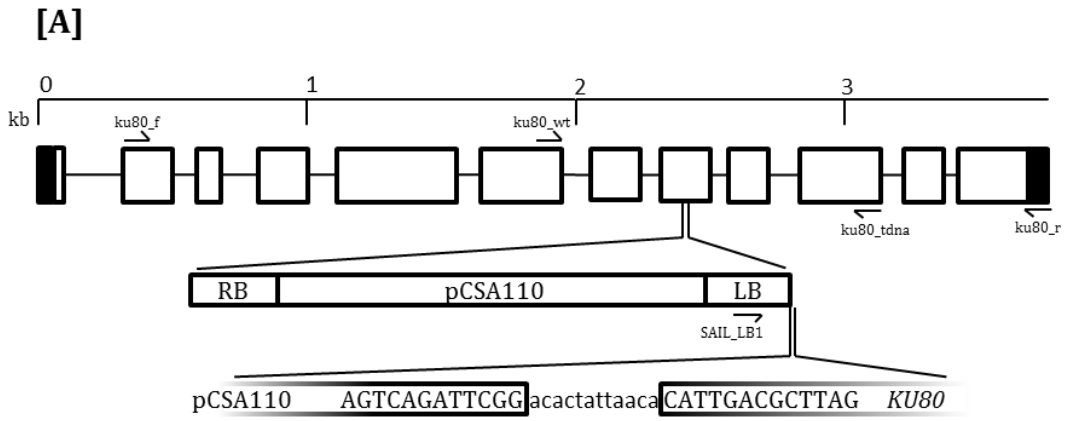


Figure 3-7 Position of *ku80-3* T-DNA insert.

[A] Schematic of the *Arabidopsis KU80* gene. Exons are denoted by boxes, with filled boxes representing UTR, empty boxes representing the coded regions and introns represented by a line. The position of the T-DNA insertion is indicated in the 8th exon. The location of the primers used for genotyping *ku80-3* are shown, with the T-DNA allele identified using primers ku80_tdna and SAIL_LB1 and the wild-type allele identified using primers ku80_tdna and ku80_wt [Table 2.2].

The presence of the T-DNA insertion was identified by the amplification of a PCR product in reactions using primers corresponding to the LB regions of the T-DNA inserts and gene specific primers as shown in [Figure 3-6 A and 3-7] for *ku70-1* and *ku80-3* respectively. The wild-type allele was detected using a pair of primers designed to the *KU70* and *KU80* genomic sequence that spanned the region of T-DNA insertion, due to the large size of the T-DNA (roughly 4 500 bp) and PCR cycle parameters, these primers were unable to amplify a PCR product in the presence of the T-DNA insertion.

The *ku70-1* (SALK_029106) mutant line has been previously described and characterised (Riha et al., 2002) and the T-DNA insertions shown to disrupt the expression of the *KU70* transcripts, however, the *ku80-3* (SAIL_714_A04) was yet to be characterised. The T-DNA PCR product for *ku80-3* was sequenced to identify the precise location of the LB of the T-DNA insertion. This result revealed the *ku80-3* T-DNA insert was located in the 8th exon [Figure 3-7].

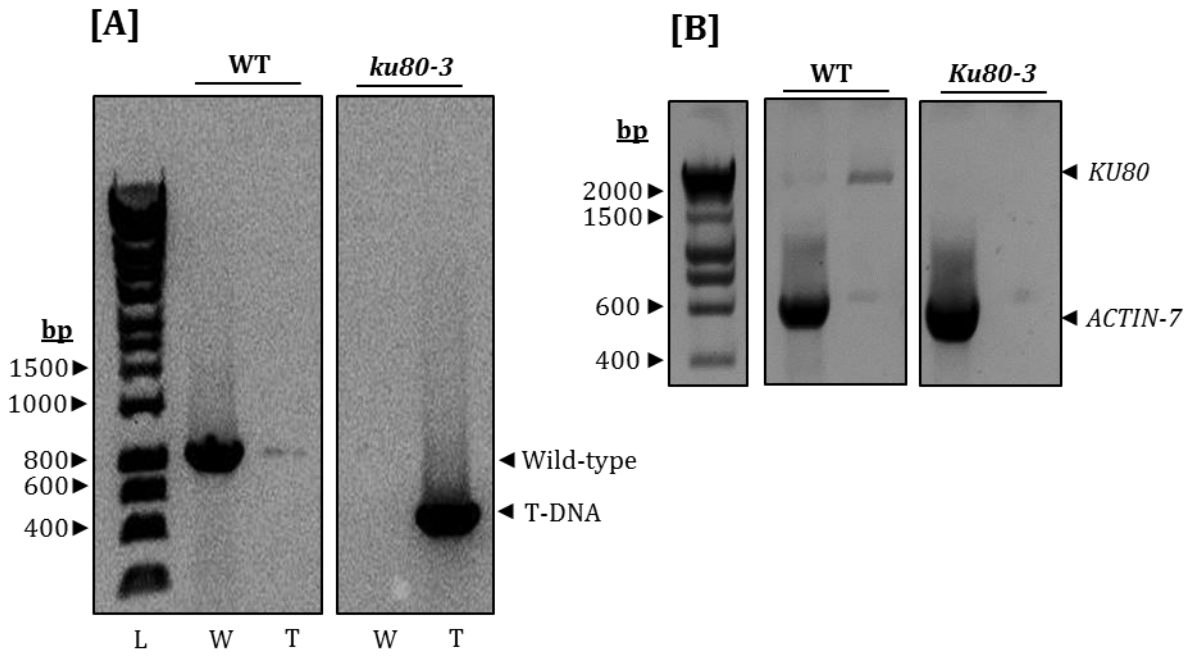


Figure 3-8 Isolation of *ku80-3* T-DNA insertion mutant.

[A] Left Border PCR of *ku80-3* T-DNA insertion. PCR based genotyping to identify wild-type (WT) and homozygous (*ku80-3*) plants for the T-DNA allele. The PCR used primers shown in Figure 4-1 [A]. The ladder used was Hyperladder 1 kb plus (lane L). Wild-type plants resulted in only a wild-type band (lane W) and homozygous plants had only the T-DNA band (lane T). PCR amplification of genomic DNA (section 2.7) was performed using DreamTaq PCR reaction mastermix (section 2.7.1) with the PCR program Taq50TD (section 2.7.2) in 20 µl.

[B] RT-PCR analysis of *KU80* transcripts in wild-type and *ku80-3* lines. Reverse transcription PCR (RT-PCR) and subsequent PCR analysis was performed as previously described (section 2.12) from wild-type and homozygous *ku80-3*. The cDNA was amplified using primers specific for *KU80* cDNA (ku80_f and ku80_r, shown in [Figure 3-8]) or using *ACTIN-7* primers as a control for cDNA synthesis [Table 2.2].

3.2.2.2 RT-PCR analysis of *KU80* transcript expression in *ku80-3*

To determine whether the T-DNA insertion in *ku80-3* lines disrupted the expression of the *KU80* transcript, reverse transcription PCR (RT-PCR) was performed using RNA isolated from 3 week old leaf tissue of homozygous *ku80-3* (section 2.10.1). The RNA was reverse transcribed to generate cDNA (section 2.10.2) and as a control for cDNA synthesis resultant cDNA was utilised in a PCR reaction to amplify a fragment of *ACTIN-7* cDNA. To identify the presence of *KU80,* PCR reactions were performed using primers which would amplify a *KU80* cDNA specific product. The forward primer used annealed to the 2nd exon of *KU80* and the reverse primer annealed to the 7th exon, creating a fragment 2,111 bp long.

In wild-type plants, a cDNA product was amplified showing the presence of the *KU80,* it was not possible to amplify a cDNA *KU80* product in the homozygous *ku80-3* line [Figure 3-8 B]. The presence of the T-DNA insertion is therefore sufficient to disrupt and prevent expression of any detectable *KU80* transcript. In both the wild-type and mutant alleles a fragment of *ACTIN-7* cDNA was amplified, demonstrating the presence of cDNA in both reactions. The *ku80-3* expression analysis was therefore consistent with this being a null mutant allele and was used for further study.

3.2.2.3 Hypersensitivity of *ku70-1* and *ku80-3* to X-ray treatment is consistent with null mutations in both genes

Growth hypersensitivity to X-ray irradiation, which induces DSBs, is widely used to provide evidence that a specific gene may have a role in DSB repair. Here wild-type, *ku70-1* and *ku80-1* seed lots were surface sterilised in 70 % ethanol for 5 min, and resuspended in dsH_2O before IR treatment. 75 or 150 Gy was delivered using a 320 kV X-ray irradiation system (NDT Equipment Services) at a rate of 1 Gy minute^{-1}. Seeds were plated individually in one row on ½ Murashige and Skoog solid medium before being stratified at 4 °C for 48 hours (section 2.5.4). The plates were then transferred to a growth chamber as previously described (section 2.4). The primary roots were measured 3, 5, and 7 days after plates had been transferred to the growth chamber and the graph displays the average root length [Figure 3-9].

To test whether there was a significant difference between the means of each genotype one-way analysis of variance (ANOVA) was used and a post hoc test (see below) implemented to determine differences between individual groups. Groups that met all assumptions of equal sample number and equal variance between the same groups were subjected to the Tukey HSD test. Groups which failed to meet either assumption used the Tukey HSD test with the Games-Howell correction to account for these violations. To test whether sample groups had equal variance the Levene's Test for Equality of Variances was applied. $P \leq 0.05$ indicated samples groups did not display equal variance.

The *ku70-1* and *ku80-3* mutant alleles displayed a wild-type growth phenotype when in optimal conditions [Figure 3-9 A]. This agreed with different previously characterised *ku80* knock-out mutants in *Arabidopsis* (West et al., 2002) and other NHEJ mutants such as *lig4* (Waterworth et al., 2010). However, when imbibed *ku70-1* and *ku80-3* seeds are subjected to X-rays, a phenotype differing from wild-type was observed [Figure 3-9 B and C] and shows statistically significant differences between the knock-out lines and wild-type root growth.

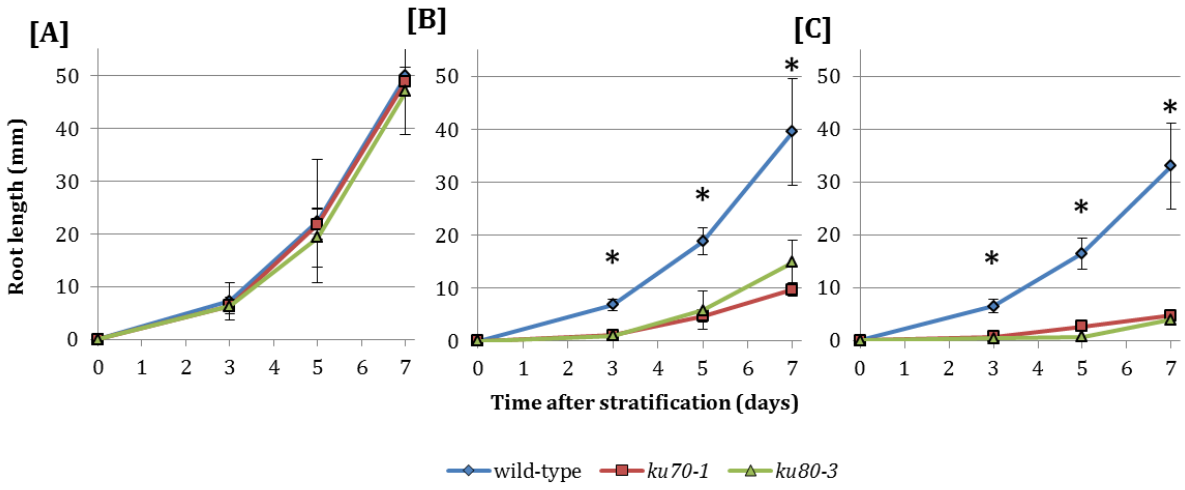


Figure 3-9 Sensitivity of *ku70-1* and *ku80-3* mutants to X-ray.

[A] Mean root length with no X-ray treatment: Wild-type, *ku70-1* and *ku80-3* lines were grown as previously described (section 2.5.2). Seeds were plated on ½ MS agar media with X-rayed and control seeds plated in quadruplicate. The mean length of primary roots is shown after 3, 5 and 7 days post imbibition (n=7). The error bars displayed are +/- standard error . The probability associated with Levene's Test for Equality of Variances at three days (p=0.382), five days (p=0.118) and seven days (p=0.031). A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD test indicated the mean score for 3 (wild-type; M = 7.37 mm SEM = 1.21, *ku70-1*; M = 6.37 mm SEM = 1.59, *ku80-3*; M = 6.26 SEM = 1.04) and 5 days growth (wild-type; M = 22.10 SEM = 3.78, *ku70-1*; M = 21.81 mm SEM = 2.86, *ku80-3*; M = 19.33 mm SEM = 5.56) were not significant between any genotype ($p > 0.05$). Post hoc comparisons using the Games-Howell correction indicate the mean scores for 7 days growth (wild-type; M = 52.40 mm SEM = 10.09, *ku70-1*; M = 48.82 mm SEM = 2.73, *ku80-3*; M = 46.95 mm SEM = 8.14) were not significant between genotypes either ($p > 0.05$).

[B] Mean root length with 75 Gy X-ray treatment: Wild-type (n = 11), *ku70-1* (n = 9) and *ku80-3* (n = 9) lines were treated with a 75 Gy dose of x-ray and root growth recorded as previously described (section 2.5.4). The probability associated with Levene's Test for Equality of Variances at 3 days (p=0.175), 5 days (p=0.116) and 7 days (p=0.022). A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD test with the Games-Howell correction indicated the means score at 3 days growth (wild-type; M = 6.81 mm SEM = 1.03, *ku70-1*; M = 1.02 mm SEM = 0.56, *ku80-3*; M = 0.99mm SEM = 0.43) are significantly different in the *ku70-1* and *ku80-3* ($p < 0.0005$) lines when compared to wild-type root growth. There was no significant difference found between the mean of the *ku70-1* and *ku80-3* ($p > 0.05$). 5 days growth (wild-type; M = 18.49 mm SEM = 2.73, *ku70-1*; M = 4.66 mm SEM = 1.78, *ku80-3*; M = 5.75 mm SEM =3.64) shows a significant difference between *ku70-1* and *ku80-3* ($p < 0.0005$) lines when compared to wild-type root growth. There was no significant difference found between the mean of the *ku70-1* and *ku80-3* ($p > 0.704$). 7 days growth (wild-type; M = 39.57 mm SEM = 9.63, *ku70-1*; M = 9.71 mm SEM = 4.97, *ku80-3*; M = 14.91 mm SEM = 4.18) shows a significant difference between *ku70-1* and *ku80-3* ($p < 0.0005$) lines when compared to wild-type root growth. There was no significant difference found between the mean of the *ku70-1* and *ku80-3* ($p = 0.071$). * indicates a $P < 0.05$ significant difference within the 3 genotype means

[C] Mean root length with 150 Gy X-ray treatment: Wild-type (n = 13), *ku70-1* (n = 7) and *ku80-3* (n = 10).). The probability associated with Levene's Test for Equality of Variances at 3 days (p=0.001), 5 days (p=0.002) and 7 days (p=0.001). A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD test with the Games-Howell correction indicated the means score at 3 days growth (wild-type; M = 6.52 mm SEM = 1.30, *ku70-1*; M = 0.71 mm SEM = 0.54, *ku80-3*; M = 0.41 mm SEM = 0.47) show significantly different mean values in the *ku70-1* and *ku80-3* ($p < 0.0005$) lines when compared to wild-type root growth. The means of *ku70-1* and *ku80-3* show no significant difference ($P = 0.485$). 5 days root growth (wild-type; M = 16.46 mm SEM = 2.91, *ku70-1*; M = 2.72 mm SEM = 0.63, *ku80-3*; M = 0.95 mm SEM = 1.73) shows a significant difference between *ku70-1* and *ku80-3* ($p < 0.0005$) lines when compared to wild-type root growth and show no significant difference between each other ($P = 0.209$). After 7 days root growth (wild-type; M = 33.09 mm SEM = 8.16, *ku70-1*; M = 4.71 mm SEM = 3.22, *ku80-3*; M = 3.89 mm SEM = 2.68) shows a significant difference between *ku70-1* and *ku80-3 3* ($P < 0.0005$) lines when compared to wild-type root growth and show no significant difference between each other ($P = 0.846$).

After a 75 Gy dose of X-rays, a reduction in root growth in both the *ku70* and *ku80* deficient lines was clearly visible when compared to wild-type. This was confirmed by the very low P-values ($P < 0.0005$) for all group mean root lengths [Figure 3-9 B]. Interestingly, there was no significant difference between the means of *ku70-1* and *ku80-3* at any measured point after 75 Gy radiation ($P > 0.05$), consistent with similar roles for *KU70* and *KU80* in protecting the *Arabidopsis* genome against DSBs. Wild-type root growth after 75 Gy was not significantly different to wild-type growth without irradiation ($P > 0.05$) at any point of measure [Figure 3-9 A and B]. After 150 Gy wild-type root growth was significantly reduced after 5 days when comparing the growth of non-irradiated wild-type seedlings [Figure 3-9 A and C]. The reduction of root growth was exacerbated in *ku70* and *ku80* mutant lines when exposed to a higher dose of X-rays, i.e. from 75 Gy to 150 Gy. There was a significant difference between root growth in both *ku70* and *ku80* mutant when compared growth between 75 and 150 Gy treatments after 5 and 7 days respectively ($P < 0.05$).

As previously reported these results confirmed that the mutant lines, including the newly isolated *ku80-3*, were hypersensitive to high energy irradiation. These lines were taken forward for analysis of seed vigour, viability and longevity. Furthermore, the fact that mammalian KU70 and KU80 function as a heterodimer (Tamura et al., 2002) and display similar hypersensitivity to X-rays allows independent assessment of the roles of the KU complex in seed physiology.

3.2.2.4 Germination performance of high quality *ku70-1* and *ku80-3* seeds shows the requirement of these genes to maintain high vigour seeds

Work has shown components of the NHEJ repair pathway to have some role seed vigour. Removal of gene function of either *LIG4* or *LIG6,* both involved in DSB repair in *Arabidopsis*, caused a delay in seed germination (Waterworth et al., 2010). LIG4 function is well characterised and central to the c-NHEJ pathway (West et al., 2000). To identify a role for other repair factors known to function in the c-NHEJ pathway, analysis of *ku70-1* and *ku80-3* germination performance was undertaken.

If *KU70* and *KU80* have roles in ensuring vigorous germination as *LIG4* and *LIG6* do, then the knock-out lines for both these genes should have shown decreased seed vigour expressed as an increased time to complete germination relative to wild-type. To determine if these genes had specific roles in seed vigour, germination assays were performed with the mutant seeds lines *ku70-1* and *ku80-3* and compared to wild-type. Forty seeds of each genotype were plated in triplicate onto germination paper (Anchor Blue, California) in a 90 mm petri dish with 7.5 ml dH_2O and stratified at 4 °C for 2 days before being moved to a growth chamber (section 2.5). Germination was scored at time points up to 100 hours and then converted into a percentage of seeds germinated from total lot relative to wild type controls. Germination was scored as protrusion of the radicle from the seed coat and high quality (section 2.5) wild-type, *ku70-1* and *ku80-3* seed lots resulted in c. 100% viability in all genotypes tested [Figure 3-10 A] .

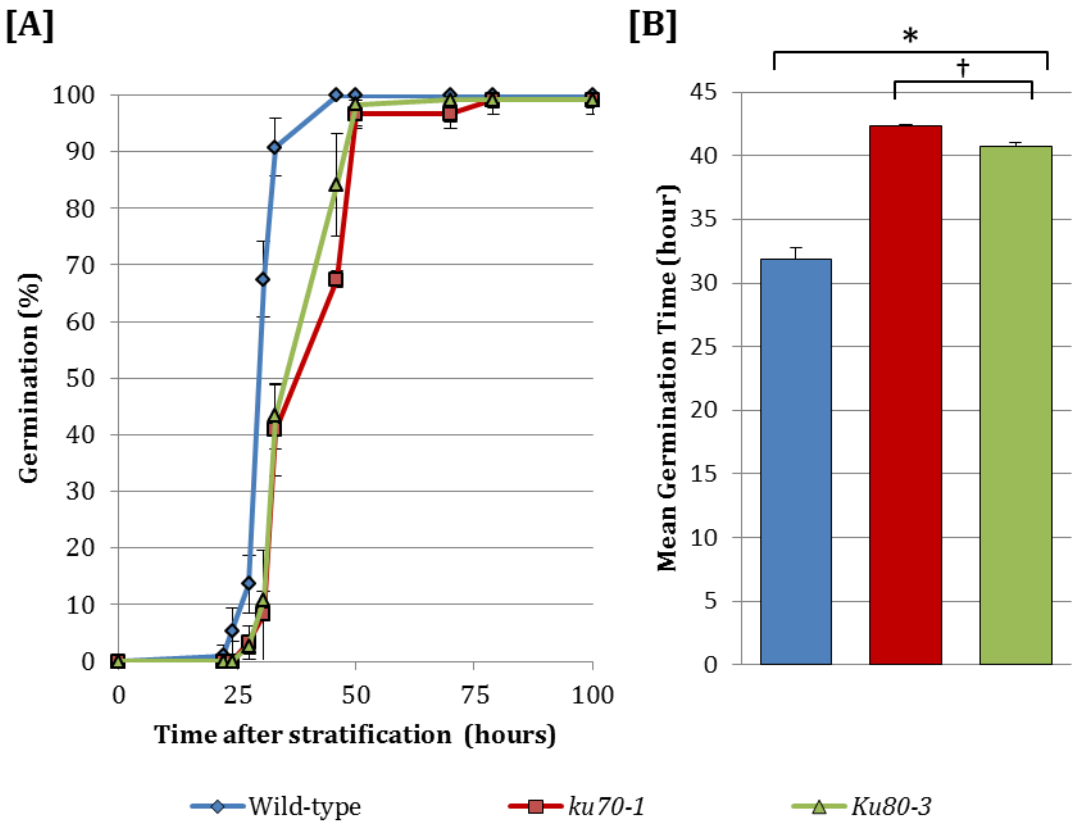


Figure 3-10 Germination performance of high quality *ku70-1* and *ku80-3* seeds.

[A] Germination of wild-type, *ku70-1* and *ku80-3* high quality seeds. Germination performance of *ku70-1* and *ku80-3* mutant seed (n=40) performed in triplicate, analysed at 23 °C under 16 hour light and 8 hour dark cycles. Error bars show the standard error. Total viability for: wild-type M = 100.00 % (SEM = 0), *ku70-1* M = 99.17 (SEM = 1.44), *ku80-3* M = 99.17 (SEM = 1.44). The probability associated with Levene's Test for Equality of Variances (p=0.02). Post hoc comparisons using the Tukey HSD test with the Games-Howell correction indicated no significant difference in final viability between any of the genotypes (P > 0.05).

[B] Mean germination time of each genotype. Mean germination time (MGT) for physiological germination is presented. Error bars show the standard error around the mean (SEM). Wild-type M = 31.91 hours (SEM = 0.83), *ku70-1* M = 42.74 hours (SEM = 0.081) *ku80-3* M = 40.75 hours (SEM = 0.32). The probability associated with Levene's Test for Equality of Variances (p=0.09), A one-way ANOVA was conducted to compare mean germination time. Post hoc comparisons using the Tukey HSD test indicated the means score for MGT was significantly different for between *ku70-1* and *ku80-3* (p < 0.0005) lines when compared to wild-type MGT, denoted by an asterisk (*). There was also a significant difference between *ku70-1* and *ku80-3* (P= 0.021), denoted by a cross (†).

Previous work also showed high viability of mutant seeds deficient in two other DSB repair factors, *lig4* and *lig6*, in the absence of seed ageing (Waterworth et al., 2010). Here, the germination performances showed a decrease in vigour (MGT), when compared to the mutant lines individually to wild-type germination performance. Post hoc analysis confirms there are significant differences between wild-type MGT and that of the mutant lines ($p < 0.0005$). Specifically, seeds deficient in either of these two c-NHEJ factors significantly slower germination than wild-type seeds. This data indicated that both *KU70* and *KU80* genes were required to maintain strong vigour in seeds of otherwise high quality. These results further supported the growing evidence from molecular analysis here, that repair factors, particularly DSB repair factors, play a crucial role maintaining high seed vigour.

Interestingly, post-hoc analysis showed a significant difference between the MGT of *ku70-1* and *ku80-3* seed lots, with *ku70-1* seeds taking significantly longer to germinate on average [Figure 3-10 B]. As phenotypic analysis showed both mutants had the same levels hypersensitivity to DNA damage [Figure 3-10], this difference in MGT might not be expected. This result could be interpreted as demonstrating *KU70* has a more important role in seed vigour than *KU80.*

3.2.2.5 Germination performance of *ku70-1* and *ku80-3* seeds after accelerated ageing treatment shows a requirement for the KU complex in seed longevity.

The delayed germination exhibited by both *ku70-1* and *ku80-3* mutants further suggested that DNA damage and repair is important to seed germination and seed quality. Seed storability (longevity) is an agromonically important aspect of seed quality (section 1.1.4). It is also important in the conservation of plant genetic resources. Increases in seed moisture content and temperature during storage results in quantifiable and predictable decreases in seed viability (Roberts, 1973). It has been shown through cytological studies that quantified chromosomal aberrations (which are products of mis-repaired DSBs) in seeds,

even small losses of seed viability through ageing are inevitably associated with an increase of chromosome damage (Dourado and Roberts, 1984).

To determine if *KU70* or *KU80* were important factors in seed longevity, low quality seeds were produced by accelerated ageing (AA). Seed lots are exposed to conditions of elevated temperature and relative humidity (Hay et al., 2003) (section 2.4). This is a widely used industry-standard technique that mimics long term storage in sub-optimal conditions (Matthews et al., 2012). If a significant decrease in seed quality was observed, in terms of either viability or vigour after AA treatment in the mutant lines, this would have indicated a role for these factors in seed longevity. Wild-type seeds were treated alongside *ku70-1* and *ku80-3* mutant seeds to form the control group.

Seeds were exposed to either 2 [Figure 3-11 A] or 4 days [Figure 3-11 B] accelerated ageing before being immediately imbibed, plated out in triplicate (n = 40) and stratified in the dark at 4 °C for 48 hours as previously described (section 2.4). Seeds were then moved to the growth cabinet (time = 0). Germination was scored daily for the first 12 days and again between 16 and 18 days.

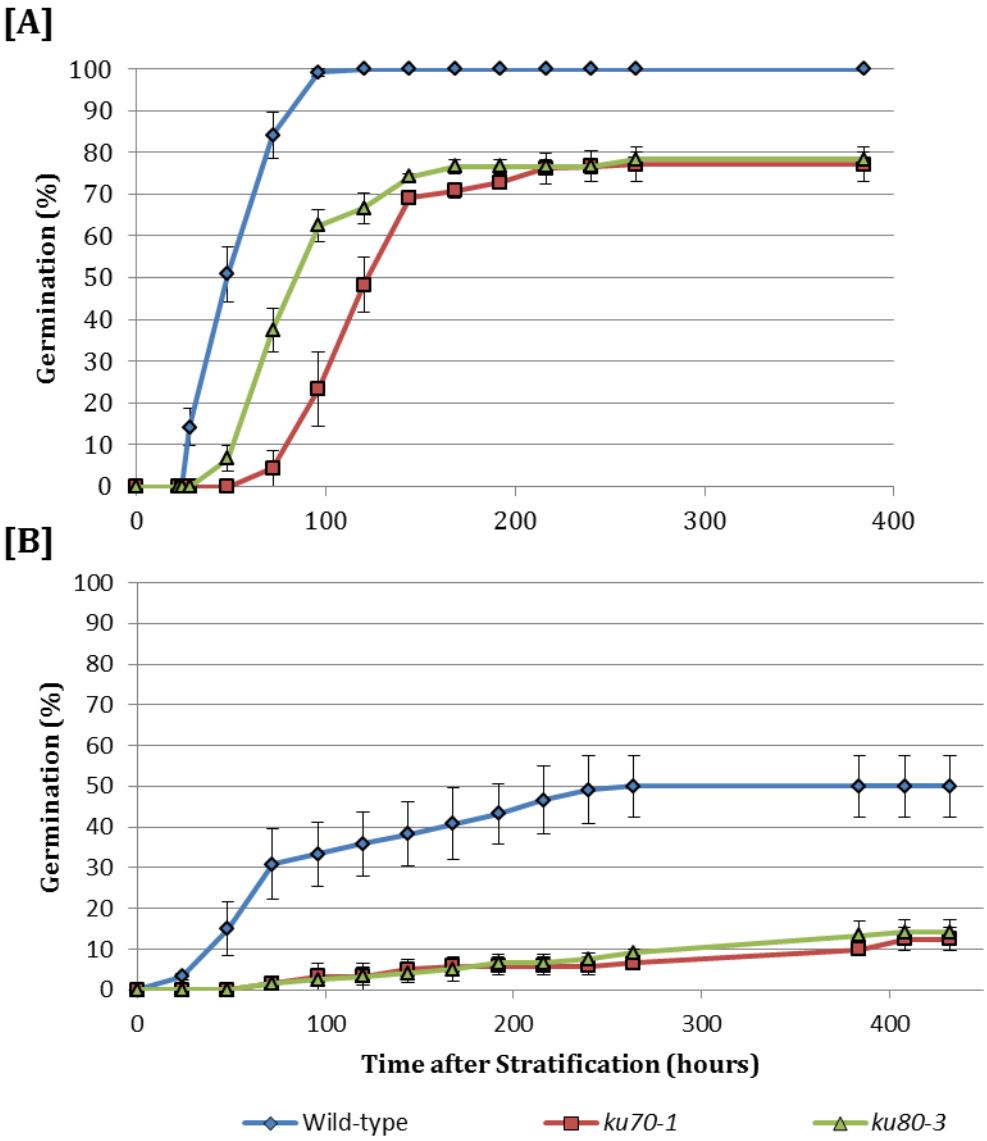


Figure 3-11 Germination performance of *ku70-1* and *ku80-3* in 2 and 4 day aged seeds (40 °C at 82% relative humidity)

[A] Comparison of Col-0 and mutant seeds mutant which have undergone 2 days accelerated ageing. Seeds underwent accelerated ageing treatment at 40 °C at 82% relative humidity for 48 hours (Winston and Bates, 1960). Seeds were immediately plated on germination paper in triplicate (n = 40) and stratified at 4 °C for 48 hours before transfer to 23 °C (time = 0 hours). Germination performance of *ku70-1* and *ku80-3* performed under 16 hour light and 8 hour dark cycles at 23°C is shown (section 2.5).

[B] Comparison of Col-0 and mutant seeds mutant which have undergone 4 days accelerated ageing Seeds underwent accelerated ageing treatment at 40 °C at 82% relative humidity for 96 hours (Winston and Bates, 1960). Germination performance analysis was undertaken as previously described in [A] (n = 40).

MGT indicated a significant decrease in vigour in the aged mutant lines relative to wild type controls [Figure 3-12 C D]. Interesting after 2 days ageing, wild-type seeds displayed no loss in viability with 100 % seeds germinating [Figure 3-12 A]. Conversely, both the mutant lines seeds failed to germinate after the 16 day final time point. Wild-type seeds did display an approximate two-fold delay in germination after ageing (61.0 hours) when compared to high quality wild-type seeds (31.9 hours) [Figures 3-12 C and 3-10 B]. The *ku70-1* and *ku80-3* mutant seeds had significantly longer MGTs (126.5 and 94.0 hours respectively) than wild-type after 2 days accelerated ageing treatment. There was a difference ~3.5-6 times greater than that observed in high quality seeds.

After 4 days ageing treatment there was a substantial difference between the mean germination times of *ku70-1* and *ku80-3* mutant seeds (276.3 and 238.8 hours) and wild-type (105.0 hours). This was an increase from approximately 10 hours between unaged mutant and wild type lines to well over 100 hours after ageing treatment. A statistically significant difference was observed in the MGT between 2 day aged *ku70-1* and *ku80-3*, which was consistent with the analysis shown in high quality seeds [Figure 3-10]. Interestingly, this significant difference was lost after *ku70-1* and *ku80-3* seeds have been aged for 4 days, although there was greater variation in mean values at this time point.

In high quality seed lots [Figure 3-10 A], there was no difference in the final viability of wild-type or either mutant line. In seeds that underwent AA treatment for 2 days a significant decrease in viability was apparent in both the *ku70-1* and *ku80-3* mutant lines when compared to wild-type with decreases of 21.5 % and 21.7 % respectively [Figure 3-12 A, B]. After the 4 day treatment, wild-type total viability had decreased by 50% but the viability of *ku70-1* and *ku80-3* mutant seeds was only 12.5 % and 14.2 %. This was a difference of 37.5 % and 35.8 %, demonstrating loss of viability is further exacerbated in the mutant seeds that have undergone prolonged AA treatment.

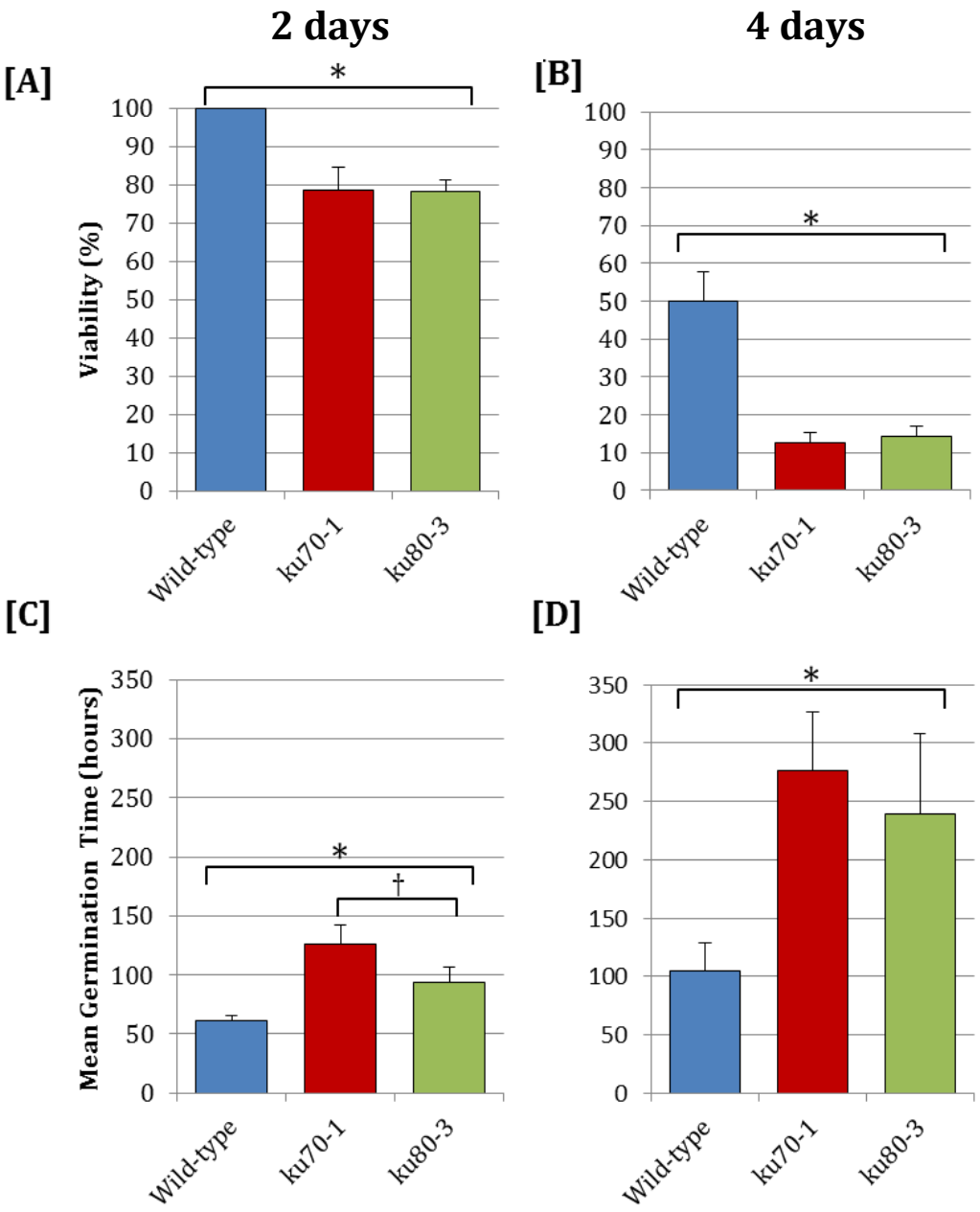


Figure 3-12 Germination performance of *ku70-1* and *ku80-3* seeds after 2 and 4 day aged (40 °C at 82% relative humidity)

[A-B] Comparison of total germination in wild-type and mutant seeds.

(A) 2 days accelerated aged seeds. Seeds underwent 2 days accelerated ageing and were allowed to germinate as previously described (section 2.5) in triplicate (n=40), the final germination score was marked after 18 days and displayed here as a percentage. Error bars show the standard error. Wild-type (M = 100.00 %, SEM = 00.00) *ku70-1* (M = 78.50 %, SEM = 6.06) and *ku80-3* (M = 78.33 %, SEM = 2.89). A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD test indicated the means for *ku70-1* and *ku80-3* are significantly different to the wild-type mean (P = 0.001), denoted by an asterisk (*). The means for *ku70-1* and *ku80*-3 are not significantly different from each other (P = 0.998).

(B) Viability of 4 days accelerated aged seed lots. Seeds underwent 4 days accelerated ageing and were allowed to germinate as previously described (section 2.5) in triplicate (n=40), the final germination score was marked after 18 days and displayed here as a percentage. Wild-type (M = 50.00 %, SEM = 13.23) *ku70-1* (M = 12.50 %, SEM = 5.00) and *ku80-3* (M = 14.17 %, SEM = 5.20) Error bars show the standard error. A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD test indicated the means for *ku70-1* and *ku80-3* are both significantly different to the wild-type mean (P = 0.004 and 0.006 respectively), denoted by an asterisk (*). The means for *ku70-1* and *ku80*-3 are not significantly different from each other (P = 0.970).

[C-D] Comparison of MGT in wild-type and mutant seeds.

(C) 2 days aged seed lots. Mean germination time (MGT) for physiological germination is presented for the germination assays of seeds that had undergone 2 days accelerated ageing with germination measured daily over 16 days. Error bars show the standard error. The graph shows MGT for Wild-type (M = 60.97hours, %, SEM = 4.64), *ku70-1* (M = 126.49 hours, SEM = 15.60) and *ku80-3* (M = 94.02 hours, SEM = 12.31)**.** A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD test indicated the means for *ku70-1* and *ku80-3* are significantly different, denoted by an asterisk (*), to the wild-type mean (P = 0.001 and 0.032 respectively). The means for *ku70-1* and *ku80*-3 are also significantly different from each other (P = 0.034).

(D) MGT of 4 days aged seed lots. Mean germination time (MGT) for physiological germination is presented for the germination assays of seeds that had undergone 2 days accelerated ageing with germination measured daily over 18 days. The graph shows MGT for Wild-type (M = 104.99 hours, SEM = 23.40), *ku70-1* (M = 276.34 hours, SEM = 49.81) and *ku80-3* (M = 238.80 hours, SEM = 69.69)**.** A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD test indicated the means for *ku70-1* and *ku80-3* are significantly different, denoted by an asterisk (*), to the wild-type mean (P = 0.015 and 0.046 respectively). The means for *ku70-1* and *ku80*-3 are not significantly different from each other (P = 0.662).

It also should be noted that there was a large increase in the spread of data for the MGT after 4 days AA. In high quality seeds neither the wild-type nor the mutant lines had a standard error (SEM) < 0.32h. However, in 4 days aged seeds, wild-type had an SEM of 23.40h and *ku70-1* and *ku80-3* mutant seeds had an SEM of 49.81h and 69.69h respectively. A greater spread in the MGT values around the average as was observed here is indicative of seeds losing synchronicity in germination. This is a defining characteristic of seed lots with low germination potential and poor seed quality (Roberts, 1973). The mutant lines both had a SEM much larger than wild-type when seeds were aged for 4 days, indicating the mutant lines seeds are of a lower quality than wild-type.

Taken together these results demonstrated the importance for *KU70* and *KU80* in maintaining seed vigour and viability. When deficient in either *ku70* or *ku80* seeds were hypersensitive to suboptimal storage conditions i.e. seeds were shown to have decreased longevity through the decrease in total seed germination.

3.2.2.6 *ku70-1* and *ku80-3* seeds lose viability after accelerated ageing treatment rather than become dormant.

Accelerated ageing caused a decrease in total germination observed [Figure 3-12]. The hypersensitivity of the treatment in these mutant lines suggested that the seed lots lose viability. It is hypothesised that the decrease of total germination in the mutant lines relative to the wild-type was due to the inability of these lines to repair accumulated damage to the genome.

It was feasible that the failure of some seeds to germinate was caused by a thermo-dormancy induced on AA treatment. Furthermore, it was a possibility that the mutant lines examined here are more susceptible to becoming dormant after storage in unfavourable conditions or if there is an altered primary or secondary dormancy in the mutant lines (section 1.1.2). To test this hypothesis, seeds which had undergone accelerated ageing were imbibed (as in section 3.2.4) and subsequently treated with the dormancy breaking chemicals (section 2.5.5).

A control experiment was performed using dormant seeds to ensuring that the dormancy-breaking treatment was effective [Figure 3-13]. Dormant wild-type, *ku70-1* and *ku80-3* seeds were produced by growing the mother plant at 15 °C, collecting the seeds without after ripening (Kendall et al., 2011) and storing at – 80 °C until required (section 2.5.1).

Dormant seeds were imbibed on germination paper and put directly into the growth chamber without stratification so as not to encourage dormancy to be broken. Germination performance was measured every 48 hours until the dormancy was released using a cocktail of gibberellic acid hormones and the ABA-synthesis inhibitor, fluridone (Bentsink et al., 2006). Dormancy release was initiated at 14 days after imbibition. Germination was recorded daily for a further 10 days (section 2.5.5).

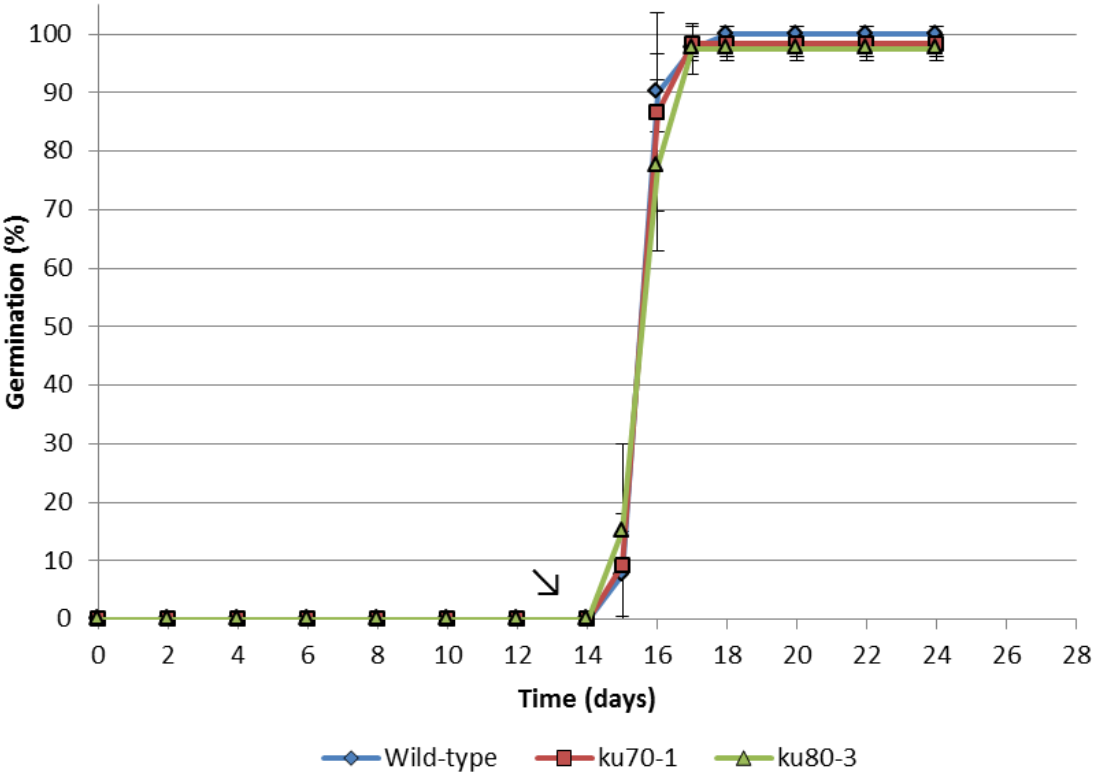


Figure 3-13 Germination performance of dormant wild-type, *ku70-1* and *ku80-3* seeds before and after dormancy breaking treatment

The germination performance of dormant wild-type, *ku70-1* and *ku80-3* seeds (section 2.5.1.2) was analysed. The germination assay was performed and recorded as previously stated with (section 2.5.2) except seeds were not stratified. After 14 days in the growth chamber, a dormancy-breaking chemical cocktail was applied (highlighted with the arrow) to the seeds (section 2.5.5). The error bars display the standard error of the mean.

The graph [Figure 3-13] showed seeds of each genotype did not germinate up to two weeks after imbibition, indicating the dormant nature of all seed lots. Upon addition of the dormancy-breaking cocktail to the dormant wild-type, *ku70-1* and *ku80-3* seed lots each germinated to near 100 % within three days. The result presented here [Figure 3-13], confirmed this treatment is effective in breaking seed dormancy.

To test the hypotheses that AA treatment induced thermodormancy in wild-type, *ku70-1* and *ku80-3* seeds or the mutant lines have altered dormancy levels, 2 and 4 day AA treated seeds were imbibed, plated on germination paper, stratified for 48 hours before being moved to the growth chamber under long day conditions (section 2.5). Germination was scored after 21 days before seed lots were treated with the dormancy-breaking chemical cocktail and left before seeds were scored again 7 days later [Figure 3-14]. No germination was induced by the dormancy-breaking treatment in any seed lot after AA.

These results did not support the hypothesis that dormancy in the different seed lines after AA treatment is the cause for the decrease in total germination [Figure 3-14]. This supported the hypothesis that the accelerated ageing caused the seeds to lose viability and was consistent with decreased longevity in *ku70-1* and *ku80-3* mutant lines.

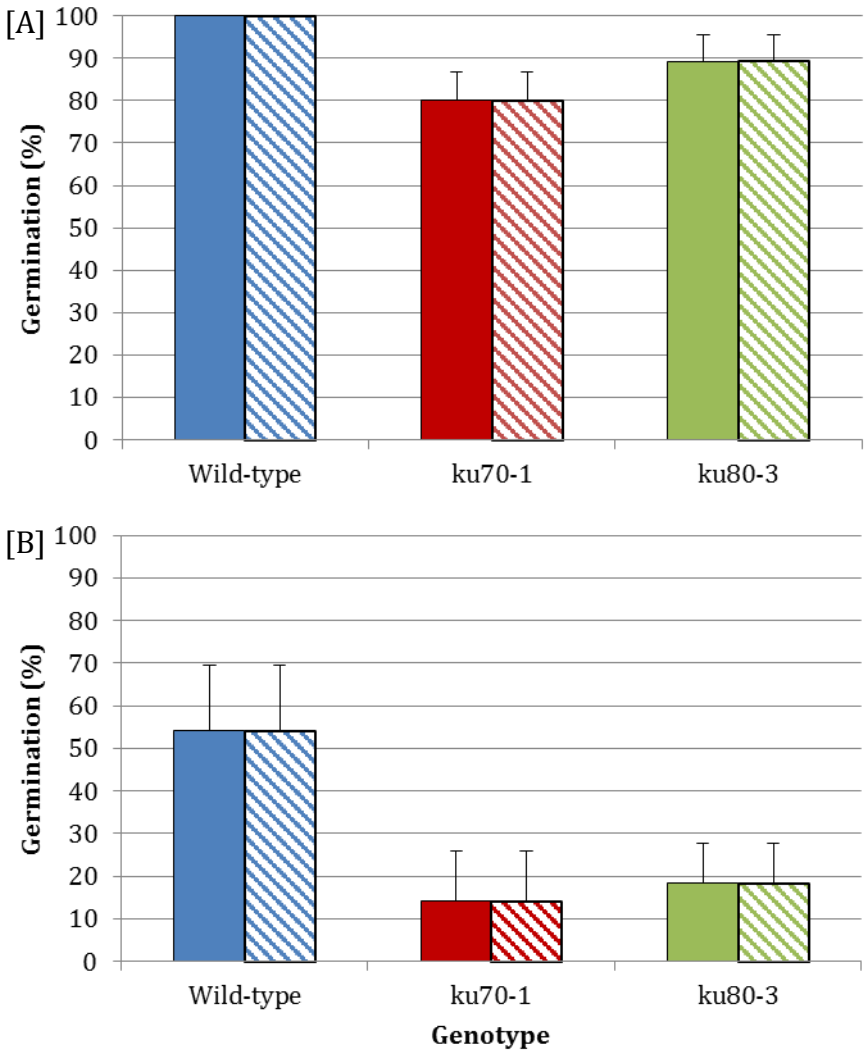


Figure 3-14 Analysis of total germination in aged wild-type, *ku70-1* and *ku80-3* seeds before and after dormancy breaking treatment

Germination performance of wild-type, *ku70-1* and *ku80-3* seeds aged for **[A] 2 days** and **[B] 4 days** at (40 °C at 82% relative humidity). Germination was scored in triplicate (n = 40), 21 days after stratification (shown in bold) before being treated with the dormancy-breaking chemical cocktail (section 2.5.5). Germination was scored again 7 days later (stripes). The error bars display the standard error of the mean.

3.2.2.7 *KU80* and *KU70* are important factors for germination vigour under cold temperature

Seeds lots of differing vigour may display high germination rates under ideal conditions, but display different germination performances under stress. Germination at low temperatures slows the emergence of the radicle substantially. Cold-stress is commonly used by the seed industry to evaluate germination rates which are more representative of field conditions often encountered by field-grown crops sown in early spring and late autumn. Molecular analysis of seeds deficient in the *lig4* and *lig6* DSB repair factors has previously identified a role for the two ligases in seed vigour under cold stress (Waterworth et al., 2010). To determine if *KU80* and *KU70* had similar roles and displayed lower vigour than wild-type in low temperature conditions, the germination performance was investigated in KU-deficient lines.

Unaged *ku70-1* and *ku80-3* mutant lines seeds were imbibed and incubated at 5 °C for 10 days after which seeds were scored for germination [Figure 3-15]. At 10 days wild-type seeds displayed high levels of germination (84.2 %), whereas only 37.5 % of *ku70-1* and 59. 2 % of *ku80-3* mutants germinated. Under optimal germination conditions all genotypes show near 100% germination 2 days after imbibition, after 48 hours stratification.

To determine whether this phenotype was related to viability or vigour, seeds were transferred to 23 °C growth conditions and germination was scored 4 days later. At this point, all genotypes showed high levels of germination, wild-type (99.2 %), *ku70-1* (95.0 %) and *ku80-3* (100.0 %). The near total completion of germination in all genotypes indicated low temperature stress influences germination vigour as opposed to seed viability and the KU-deficient lines display lower vigour than wild-type. This supported the lower MGT of *ku70-1* and *ku80-3* observed previously [Figure 3-15]. However, it should be considered that whilst low temperature stress is well established in slowing of the germination processes demonstrated by the delay in radicle emergence,

prolonged imbibition of seeds at low temperature may act as an additive source of DNA damage because of increased oxidative stress.

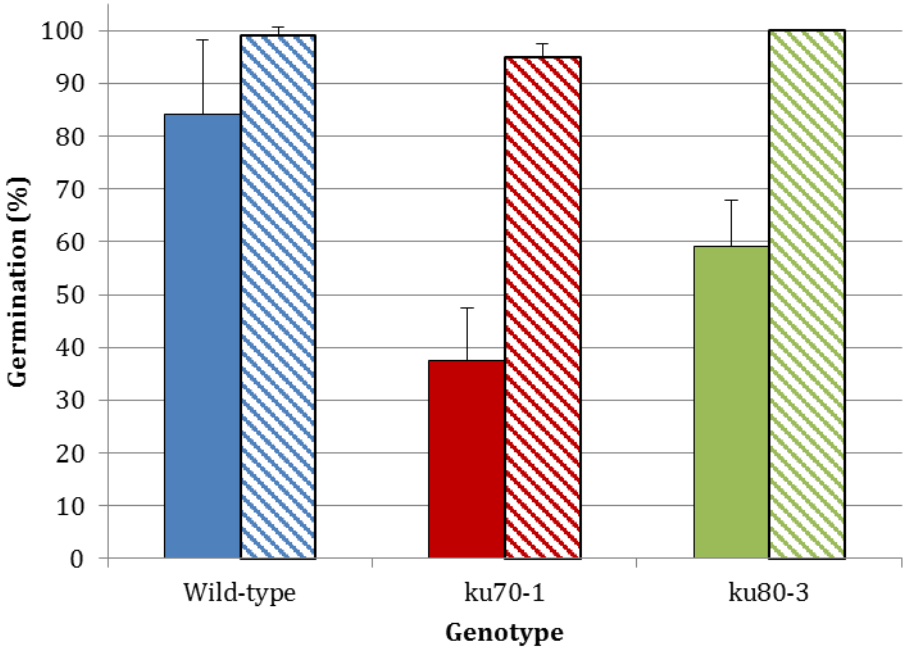


Figure 3-15 Analysis of germination in wild-type, *ku70-1* and *ku80-3* seeds under cold stress treatment

Germination performance of wild-type, *ku70-1* and *ku80-3* seeds germinated under cold stress treatment at 5 °C. Germination was scored 10 days imbibition (shown in bold). Seeds were then moved to long day conditions (section 2.5.2) and germination was scored again 4 days later. Germination was scored again 7 days later (stripes). The error bars display the standard error of the mean.

This result together with the phenotypic analysis of germination performance after each treatment supported the hypothesis that c-NHEJ repair factors are important in orthodox seed germination. This work agreed with previous reports that demonstrated the requirement for the c-NHEJ repair factor, *LIG4* in maintaining both seed vigour and longevity (Waterworth et al., 2010).

3.3 Discussion

3.3.1 Priming restores vigour but not viability in DSB repair deficient seed

Previous work had established that *Arabidopsis* seeds deficient in either *LIG4* or *LIG6* activity display a longer lag time before germination and therefore a greater MGT (Waterworth et al., 2010). In the agronomical world priming is a widely used technique to improve seed vigour in crop species to ensure seed quality and gain all the benefits associated with it (section 1.1.4.3).

Investigating DNA synthesis using the measure ^{3}H thymidine incorporation in artificially aged seeds of wild cabbage (*Brassica oleracea*) as a marker showed increased incorporation in aged seeds when compared to unaged seeds. This work also reported that treatment of these seeds with hydroxyurea (a DNA synthesis inhibitor) demonstrated that this recovery must be attributed to pre-replicative repair of DNA damage and is not linked to replication (Thornton et al., 1993). Similar studies in leek (*Allium porrum L.*) demonstrated that the correlation of DNA repair synthesis with improved germination after priming was observed across plant species (Ashraf and Bray, 1993). More recent work has shown that DNA replication in *Arabidopsis* occurs just before radicle emergence (Barrôco et al., 2005; Masubelele et al., 2005), demonstrating that DNA synthesis linked to priming is not related to DNA replication.

The work reported here showed that priming negates the effect of reduced repair capacity in *lig4* and *lig6* deficient *Arabidopsis* seeds, where onset of germination was delayed in even high quality seeds (Waterworth et al., 2010). This demonstrated further that the back-up repair pathways are sufficient to repair accumulated damage in high quality seeds. However, priming treatments themselves exert osmotic stress on the seed (section 1.1.4.3). Whilst MGT values were decreased after osmopriming treatment, overall viability was reduced in aged seeds with reduced repair capacity [Figure 3-4 and 3-5]. These results agreed with the hypothesis that repair capacity is not only important for seed vigour, but also longevity. However, it is important to note that this does

not rule out a second hypothesis that priming simply causes the advancement of germination regardless of DNA quality.

3.3.2 KU70 and KU80 are required for high seed quality in *Arabidopsis*

This work supported the hypothesis that DNA DSB repair factors were required in orthodox seeds for rapid and uniform germination. Viability and vigour has long been known to decrease upon seed ageing, with a concomitant accumulation of DNA strand breaks (Cheah and Osborne, 1978).

Previous work has shown mutant seeds deficient in *ku70* exhibit hypersensitivity to the genotoxin Methyl methanesulfonate (MMS) which causes methylation damage to bases (Riha et al., 2002). MMS-induced damage is mostly repaired by SSB repair mechanisms that excise the damaged base. If left at an intermediate step in the repair process, the SSB intermediate has the potential to generate DSBs through conversion of a SSB into a DSB during replication. In *Arabidopsis*, this hypersensitivity to MMS in *ku70*-deficient mutants was only evident during the seed stage of the plant lifecycle (Riha et al., 2002). This indicates a role for the *KU70* gene in maintenance of seed quality. However, MMS is a chemical genotoxin and may not be representative of the endogenous and exogenous stresses presented to seeds within soil.

3.3.3 KU70 and KU80 function is a determining factor of seed longevity in *Arabidopsis*

Work in the pre-genomic era demonstrated the correlation between DSB DNA damage after ageing and seed quality. Higher levels of chromosomal aberrations were observed in *Hordeum distichum* (barley) and *Pisum sativum* (pea) seeds after ageing (Dourado and Roberts, 1984). This corresponded to the mis-joining broken chromosomes after a DSB event. Subsequent molecular identification of repair processes in *Arabidopsis* have demonstrated that NHEJ repairs the majority of DSBs in non-dividing plant cells (Waterworth et al.,

2011). This random end-joining pathway revealed the mechanism by which mis-joining broken chromosomes are produced after accumulated DSBs events during sub-optimal storage and seed ageing treatments. This suggested an important role for NHEJ in seed genome repair.

The work reported in this chapter has demonstrated that both *KU70* and *KU80* were required for optimal germination vigour, even when the seeds were of high quality and stored in optimal conditions. These findings were broadly in line with the effects on seed quality after removing a functional *LIG6* in *Arabidopsis*, which was the first reported molecular link between DSB repair and seed vigour and longevity (Waterworth et al., 2010). Loss of either *ku70* or *ku80* was sufficient to decrease seed longevity as demonstrated by both mutant lines' hypersensitivity to accelerated ageing [Figure 3-11]. When compared with wild-type the two mutant lines displayed increased desynchronised germination, a factor that also correlates with loss in seed quality (Matthews et al., 2012). Upon seed ageing of the mutant lines, germination time significantly increased [Figure 3-12 C and D] and viability substantially decreased, which is indicative of reduced seed quality (Matthews et al., 2012).

3.3.4 Dormancy is not responsible for decreases in germination potential

Dormancy mechanisms have evolved to inhibit germination in response to unfavourable conditions conducive to seedling establishment (section 1.1.2). Work in *Lactuca sativa* (lettuce) seeds showed that after heat treatment for considerable lengths of time just before imbibition causes reduced germination due to increased levels of dormancy. These seeds could however be effectively released from dormancy when dormancy-releasing chemicals were applied during imbibition. (Fu and Yang, 1983). It was possible that the decreased germination potential in aged seeds related to thermo-induced dormancy associated with accelerated ageing. To test this, controls run against the aged seeds which demonstrated that an effective dormancy-breaking treatment was not able to causes the remaining quiescent seeds to germination [Figure 3-14].

When seeds were treated with a dormancy-breaking chemical cocktail, both unaged and aged mutant showed no significant difference in the number of seeds germinated than before treatment [Figure 3-14]. This work demonstrates that germination potential was not decreased in these mutant lines due to a potential increase of dormancy levels. This finding also supported the hypothesis that these c-NHEJ factors were important to seed germination via genome protection. A decrease in overall germination potential was not associated with deep-dormancy induction brought on by the accelerated ageing programme utilised.

3.3.5 Understanding the molecular responses to DSBs and controls of NHEJ as a determining factor of seed quality

Reduction in NHEJ repair capacity exhibited in the mutants studied here correlates with an overall reduced seed quality. Therefore, this work was consistence with the hypothesis that if DNA damage is not efficiently repaired upon rehydration, it can cause a loss in seed vigour (caused by an extended period of time required to repair the accumulation of damage to the genome in the seed) and eventual seed viability (when accumulation of DNA damage exceeds repair capacity). Understanding the mechanisms by which DSBs are detected in seeds may provide further insight into the importance of NHEJ in maintaining good seed quality.

Any controlling mechanisms between a decrease in seed vigour and accumulation of DSBs remains elusive. The DSB response in plants is coordinated by (section 1.6.1). *ATM*, the master kinase activated by DNA damage (Culligan et al., 2006), may control the hitherto unknown mechanisms by which seed vigour is decreased after ageing in response to accumulation of DSBs. *ATM* also regulates programmed cell death (PCD) events which occur in cells where DNA damage exceeds repair capacity (Fulcher and Sablowski, 2009). It is possible that excessive PCD events in the seed embryo, in response

to increased DNA damage accrued during ageing impedes germination and causes eventual loss of viability.

It is not conclusively known if loss of seed viability is an active choice i.e. the seed 'chooses' to die in a programmed signalling-mediated response, or passive, whereby accumulated molecular damage overwhelms the seed and it is not able to restore metabolic activity. However, work in pea has shown that increased inter-nucleosomal DNA fragmentation is observed during seed ageing which suggests that cell death is triggered after ageing (Kranner et al., 2011). Inter-nucleosomal DNA fragmentation is an indicator of active programmed cell death in plants and can be visualised as banding on an agarose electrophoresis gel (Kuthanova et al., 2008). Isolating the molecular components that regulate possible choices between repair of DSBs or cell death is important in understanding how the seed is able to repair the genome upon imbibition.

4. Characterisation of the role of homologous recombination in *Arabidopsis* seed germination

4.1 Introduction

Unlike the repair products of non-homologous end joining (NHEJ) pathway, homologous recombination (HR) repair generally preserves the integrity and fidelity of genomic material when repairing a chromosomal break. In higher plants, HR mediated repair of DSBs, is relatively rare in somatic tissues. In these cases preferential use of c-NHEJ and back-up NHEJ pathways are observed (Charbonnel et al., 2011). HR is also the recombination mechanism utilised in meiosis and there is an increased activity of HR during S-phase of mitosis (Waterworth et al., 2011). Work in tobacco has demonstrated through recombination studies of ectopic homologous sequences (not at equivalent positions on homologous chromosomes) that HR only accounts for *c.* 0.01 % of repair events in such circumstances (Puchta, 1999). Intra-chromosomal recombination assays have shown HR is much more frequent than observed in genomic DNA, with up to a third of repair events being mediated are the HR sub-pathway single strand annealing (SSA) in cases where a break is flanked by a repeated sequence (Siebert and Puchta, 2002).

4.1.1 Homologous recombination and XRCC2

The genes involved in eukaryotic HR were first identified in budding yeast and mainly belong to the *RAD52* epistasis group including *RAD51* (Pâques and Haber, 1999). The identification of homologues in other species including plants indicates that the HR repair pathway is highly conserved (Bleuyard et al., 2006). Core HR proteins include members of the *RAD51* family including *DMC1, RAD51* and five *RAD51* paralogues: *XRCC2, XRCC3, RAD51B, RAD51C* and *RAD51D*. In mammals, the five paralogues share 20–30% identity at amino acid level with RAD51 and with each other (Masson et al., 2001). Biochemical studies of the mammalian paralogue proteins have shown they form different complexes with each other: RAD51B-RAD51C-RAD51D-XRCC2, RAD51C-RAD51D-XRCC2, RAD51C-RAD51B, RAD51C-XRCC3, and RAD51C-RAD51D (Suwaki et al., 2011). In the animal model systems, mutations in the RAD51 paralogue genes are embryo-lethal (Pittman and Schimenti, 2000), so very little

in vivo data is available and therefore the functions of these various sub-complexes are still unclear.

Work in mammalian cell culture has demonstrated that XRCC2 is important for the accumulation of RAD51 (involved in the search for homology and strand pairing stages of the process) section 1.5.2) at the site of DNA damage. The loss of XRCC2 also results in a severe delay in the early response of RAD51 to DNA damage (Tambini et al., 2010).

Each of these RAD51 paralogues, including XRCC2 have been characterised in *Arabidopsis*. By contrast to their mammalian orthologues, none of the *Arabidopsis* RAD51 paralogues are required for survival in individual single mutants. (Da Ines et al., 2013; Wang et al., 2014).

The essential role of HR recombination in meiosis was a limiting factor in the studying the role of this pathway in seed longevity, vigour and viability. *Arabidopsis* lines deficient in HR factors such as *DMC1, RAD51, RAD51c* and *XRCC3* are hypersensitive to DNA damaging agents and sterile with striking meiotic chromosome fragmentation, and therefore do not produce seeds (Wang et al., 2014). However, *XRCC2* is involved in somatic DSB DNA repair but *Arabidopsis* plants deficient in this factor do not display sterility, unlike the previously mentioned HR factors (Bleuyard et al., 2005). This factor was therefore evidently dispensable for meiotic HR.

Whilst *xrcc2*-deficient plants do not display growth hypersensitivity following γ-irradiation, they showed a slower DSB repair kinetics when monitoring γ-H2AX nuclear foci formation, a widely used biomarker for DSB. The emergence and elimination of γ-H2AX foci can be used for quantitative analysis of the breakage and repair of cellular DNA (Kinner et al., 2008). In *xrcc2*-deficient plants γ-H2AX foci show slower repair than control plants (Bleuyard et al., 2005). Mutant *xrcc2* lines also exhibited a small but statistically significant increased sensitivity to the DNA cross-linking agents mitomycin C and cisplatin. In addition, hypersensitivity to methyl methanesulfonate (MMS), a chemical that causes methylation damage to bases, was also reported (Wang et al., 2014).

This showed that *XRCC2* in *Arabidopsis* is required to repair DNA cross-links but is not essential for the repair of DSBs, presumably due to the repair of DSBs by NHEJ. Comet assay analysis after bleomycin treatment (a DSB-inducing chemical) showed *Arabidopsis* plants deficient in *XRCC2* display hypersensitivity. The work also demonstrated partially redundant function of *XRCC2* with *RAD51B* and *RAD51D* as DNA fragmentation (defined as percentage of DNA in the comet tail) increased in the double and triple mutants lines (Wang et al., 2014).

4.1.2 Aims

The work already shown in chapter 3 demonstrated the importance of the DSB repair pathway NHEJ in seed vigour and longevity. DSBs are also repaired by homologous recombination in *Arabidopsis*. Therefore HR may also have a role in seed quality. Little work in the role of homologous recombination factors in germination has been reported to date. To circumvent seed production arising from the sterility of HR deficient lines, *XRCC2* was selected as the representative core HR gene to evaluate the importance and function of this recombination mechanism in seed germination and seed quality. The work presented here demonstrates that the HR gene *XRCC2* contributed to maintenance vigour in aged seeds but not overall viability.

4.2 Results

The pivotal role of homologous recombination in meiosis means that HR mutants are often sterile. *XRCC2* was well characterised with important roles in DSB repair in somatic cells with similar phenotypes to other HR mutants except it had the advantage that it was fertile (section 4.1.1). Mutant knockout *xrcc2* lines show increased sensitivity to DSBs in somatic cells but did not have an essential role in meiosis (Tambini et al., 2010; Da Ines et al., 2013; Wang et al., 2014).

4.2.1 Isolation of an XRCC2-deficient line

To determine if HR might play in germination and seed quality a T-DNA insertion line was isolated in *XRCC2* (AT5G64520). A previously well characterised T-DNA insertion for *XRCC2* designated *xrcc2-1* was identified using the SIGNAL flanking sequence tag database (http://signal.salk.edu/cgi-bin/tdnaexpress) and ordered through the Nottingham *Arabidopsis* Stock Centre (NASC, http://*Arabidopsis*.info) (Scholl et al., 2000) (section 2.3) (Tambini et al., 2010; Da Ines et al., 2013; Wang et al., 2014). The *xrcc2-1* mutant line originated from the SALK T-DNA mutant collection (Alonso et al., 2003). Primers for the mutant lines were designated xrcc2_wt and xrcc2_tdna (SALK_029106) [Figure 4-1 A].

The presence of the T-DNA *xrcc2-1* alleles was identified by the amplification of a PCR product in reactions using primers corresponding to the LB regions of the T-DNA inserts and gene specific primers as shown in [Figure 4-1 A]. The wild-type allele was detected using a pair of primers designed to the *XRCC2* genomic sequence that spanned the region of T-DNA insertion. The *xrcc2-1* (SALK_029106) mutant allele has been previously characterised (Bleuyard et al., 2005) and the T-DNA insertion was shown to disrupt gene expression.

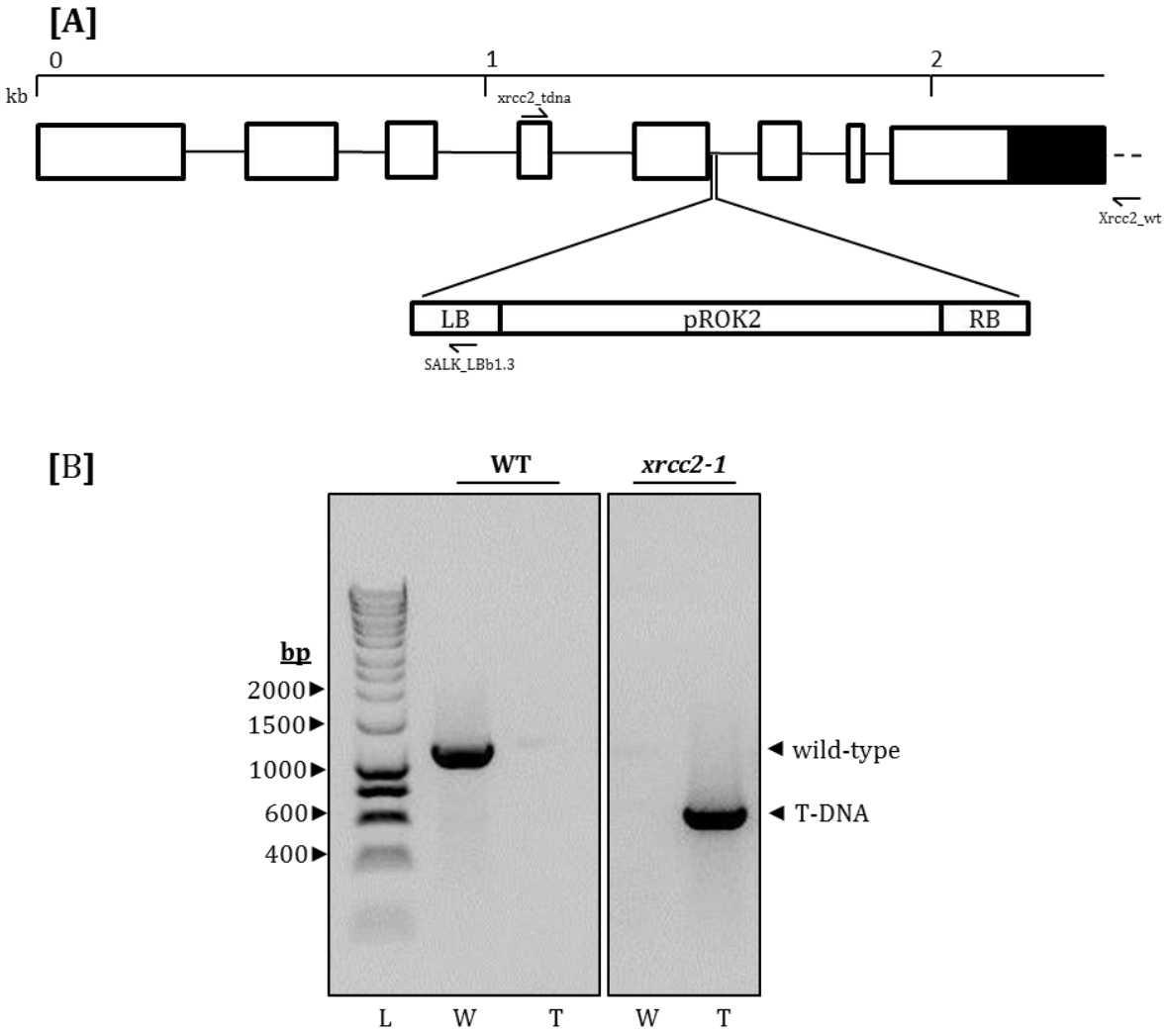


Figure 4-1 Isolation of the *xrcc2-1* T-DNA insertion mutant

[A] Schematic of the *Arabidopsis XRCC2* gene. Exons are denoted by boxes, with filled boxes representing UTR, empty boxes representing the coded regions and introns represented by a line, dashed line represent DNA outside of the gene. The position of the T-DNA insertion is indicated in the 5th intron as previously described (Bleuyard et al., 2005). The location of the primers used for genotyping *xrcc2-1* lines are shown, with the T-DNA allele identified using primers xrcc2_tdna and SALK_LBb1.3 and the wild-type allele identified using primers xrcc2_tdna and xrcc2_wt.

[B] Left Border PCR of *xrcc2-1* T-DNA insertion. PCR based genotyping to identify wild-type (WT) and homozygous *xrcc2-1* plants for the T-DNA allele. The PCR used primers shown in Figure 4-1 [A]. The ladder used was Hyperladder 1 kb plus (Bioline) (lane L). Wild-type plants resulted in only a wild-type band (lane W) and homozygous plants had only the T-DNA band (lane T). PCR amplification of genomic DNA (section 2.7) was performed using DreamTaq PCR reaction mastermix (section 2.7.1) with the PCR program Taq55TD (section 2.7.2) in 20 µl.

4.2.2 Germination assays of high quality *xrcc2-1* seeds

A wild-type phenotype has been reported for plant growth in the *xrcc2-1* mutant when grown under optimal conditions (Bleuyard et al., 2005), although germination performance was not investigated. If XRCC2 functioned to facilitate germination vigour as was observed in NHEJ-deficient mutants (section 3.2.2), then *xrcc2* mutants [Figure 4-1] should have shown delayed radicle emergence.

The germination performance of high quality seeds (section 2.5) was investigated as previously described (section 2.5.2). Wild-type and *xrcc2-1* seed germination was scored up to 100 hours imbibition [Figure 4-2]. Germination performance of *xrcc2-1* mutant seeds was not significantly different wild-type. Statistical analysis using a two-tailed Students T-test indicated no significant difference in total germination ($P = 0.374$) of the average time to germination of the two seed lots ($P=0.295$).

This result contrasts with that found in the c-NHEJ deficient mutant seed lines. This suggested those factors involved in the c-NHEJ pathway are more important to seed quality than the HR factor, *XRCC2.*

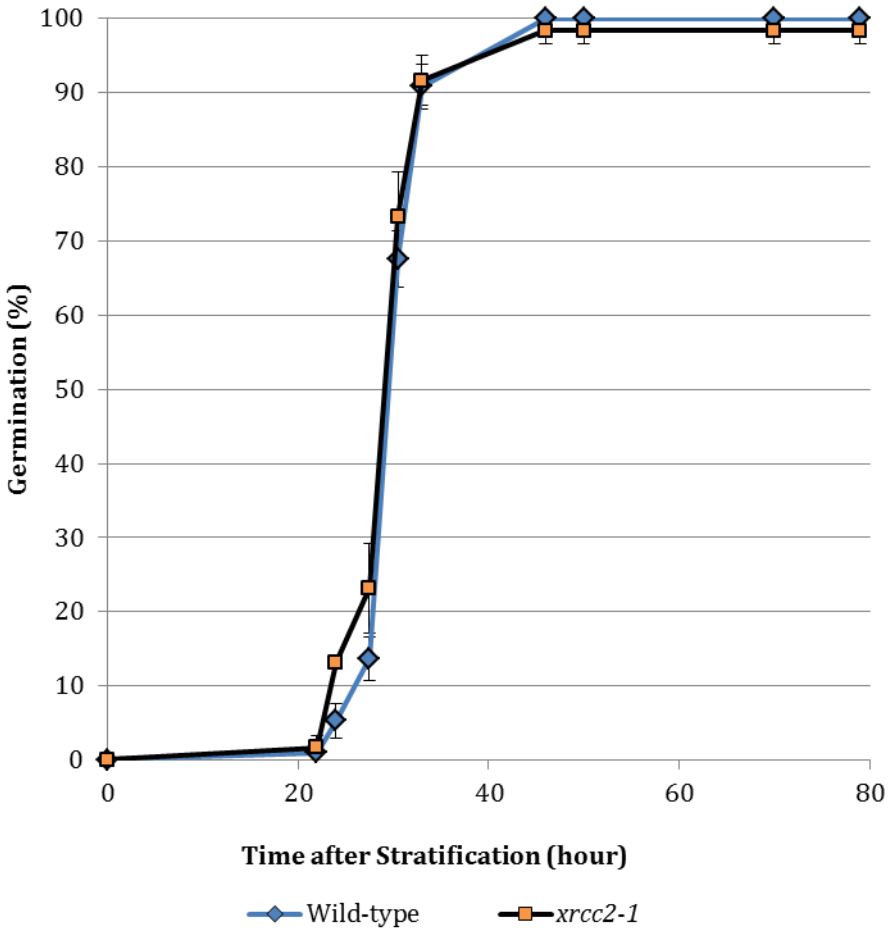


Figure 4-2 Germination profile of high quality *xrcc2-1* seeds

[A] Comparison of Col-0 and *xrcc2*-1 mutant seeds Germination performance analysed at 23 °C under 16 hour light and 8 hour dark cycles. Seeds were plated on germination paper and stratified at 4 °C for 48 hours before transfer to 23 °C (section 2.4). Total germination of wild-type was 100 % and 98.3 % in *xrcc2*-1 mutant lines (P=0.374 using Student's T-test). The MGT of wild-type was 31.91 hours and in in *xrcc2*-1 mutant lines was 30.78 hours (P = 0.295 using Student's T-test).

4.2.3 Accelerated ageing of xrcc2-1 seeds causes significant decreases in vigour but not viability relative to wild type controls

The c-NHEJ pathway is the predominant pathway for repairing DSBs in *Arabidopsis* (Charbonnel et al., 2011). This may be a factor as to why *xrcc2-1* seeds showed a wild-type germination phenotype in high quality seeds. Low levels of DSB DNA damage present in the cell were most likely repaired by the preferred c-NHEJ pathway. To test if *XRCC2*-mediated HR was required in seeds which have accumulated higher levels of DNA damage accelerated ageing was performed (section 1.1.4.2).

Seeds were exposed to either 2 [Figure 4-3 A] or 4 days [Figure 4-3 B] accelerated ageing at 40 °C and 82 % relative humidity (Winston and Bates, 1960) before being immediately imbibed, plated out in triplicate (n = 40) and stratified in the dark at 4 °C for 48 hours as previously described (section 2.4 and 2.5). Seeds were then moved to the growth cabinet (time = 0). Germination was scored every 24 hours for the first 12 days and again between 16 and 18 days.

There was no significant difference in mean total germination percentages between wild-type and *xrcc2-1* seeds after 2 days ageing treatment (P = 0.12) or 4 days ageing treatment (P = 0.82). Therefore *xrcc2-1* displayed wild-type viability even after losing 50 % of its germination potential. This differs from the phenotype observed in NHEJ-deficient mutants, where *lig4-5* (Waterworth et al., 2010), *ku70-1* and *ku80-3* (section 3.2.2.5), which displayed significantly lower levels of viability as well as a higher MGT.

A decrease in vigour in the *xrcc2-1* mutant line relative to wild-type germination performance was observed after both 2 and 4 days accelerated ageing. The *xrcc2*-1 mutant lines show a significant increase in MGT when compared to wild-type for both 2 and 4 days ageing; statistical analysis using the Student's T-test gave P values of 0.008 and 0.005 respectively [Figure 4-4 C and D].

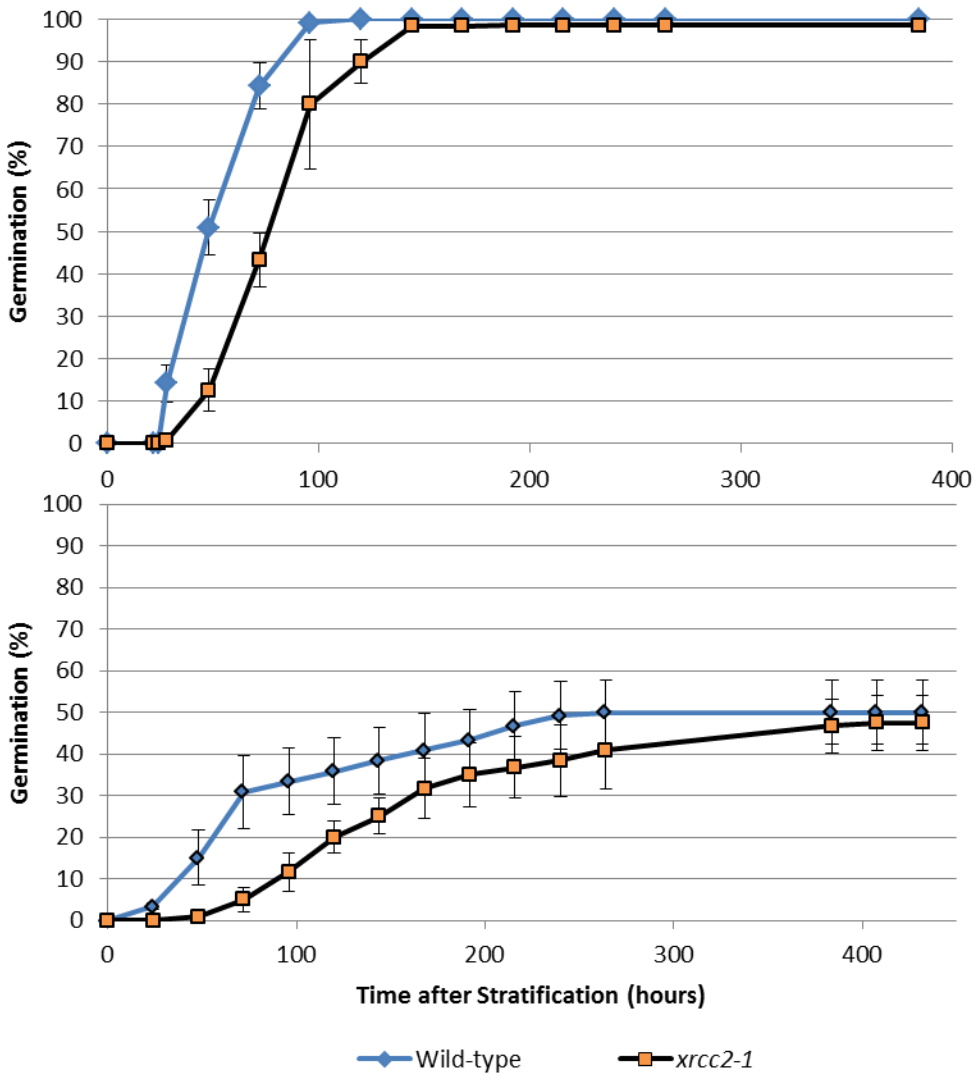


Figure 4-3 Germination profile of *xrcc2-1* seeds which have undergone accelerated ageing

[A] Comparison of Col-0 and *xrcc2-1* seeds mutant which have undergone 2 days accelerated ageing. Germination performance of *xrcc2-1* seeds analysed at 23 °C under 16 hour light and 8 hour dark cycles. Seeds were plated on germination paper and stratified at 4 °C for 48 hours in triplicate (n = 40), before transfer to 23 °C (section 2.5).

[B] Comparison of Col-0 and *xrcc2-1* seeds mutant which have undergone 4 days accelerated ageing. Seeds underwent accelerated ageing treatment at 40 °C at relatively high humidity for 96 hours. Germination performance analysis was undertaken as previously described in **[A]**.

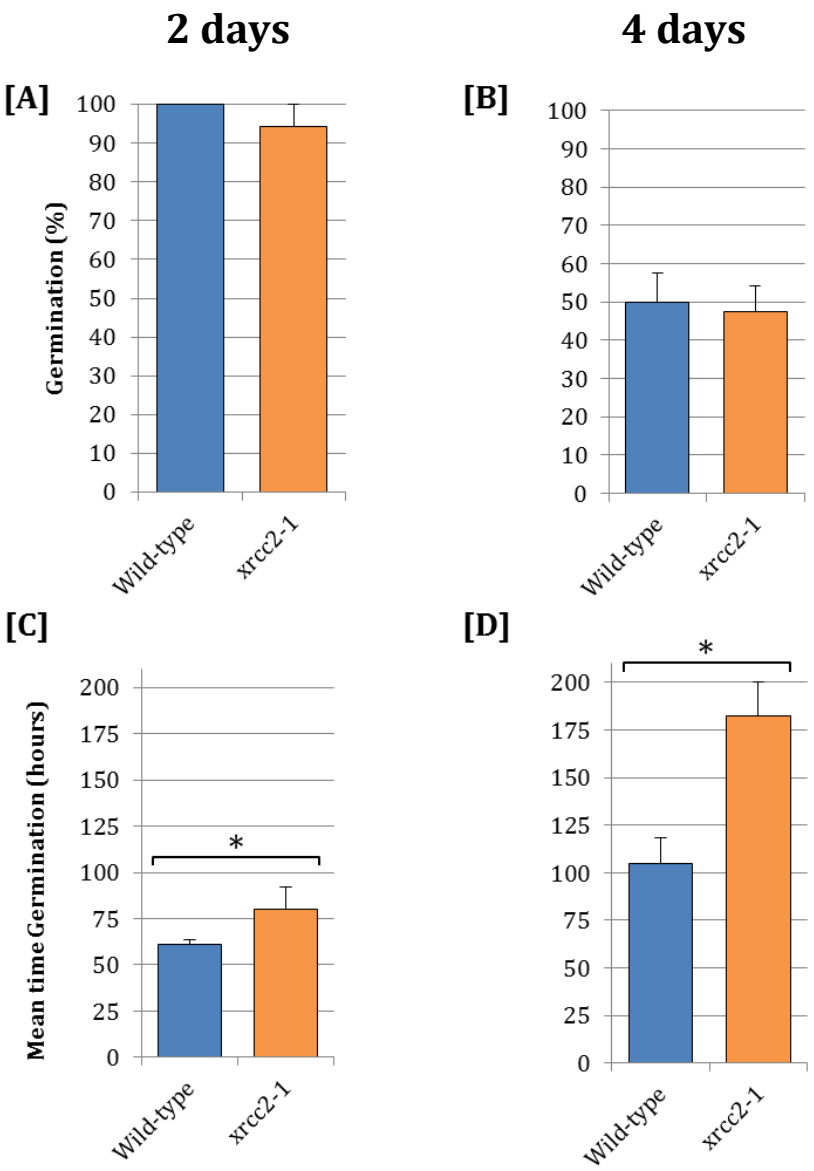


Figure 4-4 Germination profile of *xrcc2-1* seeds that have undergone accelerated ageing

[A-B] Comparison of viability in wild-type and mutant seeds mutant of (A) 2 days accelerated ageing. Germination performance of *xrcc1-2* seeds analysed at 23 °C under 16 hour light and 8 hour dark cycles. Seeds were plated on germination paper and stratified at 4 °C for 48 hours before transfer to 23 °C (section 2.5) in triplicate (n = 40), the final germination score was marked after 18 days and displayed here as a percentage with Wild-type (M = 100.00 %, SEM = 00.00) and *xrcc2-1*(M = 94.17 %, SEM = 5.77). Error bars show the standard error of the mean. Statistical analysis using the Students T-test indicates there is no significant difference between the means P = 0.16). **(B) Seeds which have undergone accelerated ageing**. Germination performance of 4 day aged *xrcc1-2* seeds analysed in triplicate (n = 40) as before. Wild-type (M = 50.00 %, SEM = 7.64) and *xrcc2-1* (M = 47.5 %, SEM = 6.61). Error bars show the standard error of the mean. Statistical analysis using the Students T-test indicates there is no significant difference between the means P = 0.82).

[C-D] Comparison of mean time germination in wild-type and *xrcc2-1* mutant seeds of (C) 2 days accelerated ageing. 2 days accelerated aged seed lots. Mean germination time (MGT)

for physiological germination is presented for the germination assays of seeds that had undergone 2 days accelerated ageing with germination measured daily over 16 days. Error bars show the standard error of the mean. The graph shows MGT for wild-type (60.97 h, SEM = 4.64) and *xrcc2-1* (M = 87.89 h, SEM = 8.17). Statistical analysis using the Students T-test indicates there is significant difference between the means (P = 0.008). **(D) Seeds which have undergone 4 accelerated ageing**. The MGT of 4 days accelerated aged seed lots. Mean germination time (MGT) for physiological germination is presented for the germination assays of seeds that had undergone 2 days accelerated ageing with germination measured daily over 18 days . The graph shows MGT for wild-type (105.0 h, SEM = 13.51) and *xrcc2-1* (M = 182.63 h, SEM = 17.75). Error bars show the standard error of the mean. Statistical analysis using the Students T-test indicates there is significant difference between the means (P = 0.005).

4.3 Discussion

4.3.1 Molecular analysis of the *xrcc2-1* mutant lines

T-DNA mutant lines were isolated to allow a reverse genetics approach to determine the function of the core HR DNA repair gene *XRCC2* in seed germination, longevity and vigour. The mutant line was predicted to have a T-DNA insertion within the respective gene at the beginning of the 5th intron: the *xrcc2-1* line has been previously characterised (Tambini et al., 2010; Da Ines et al., 2013). PCR-based genotyping was used here to confirm that a homozygous line of the characterised mutant allele was used in the present study.

4.3.2 Germination vigour is not changed in *xrcc2-1* deficient high quality seeds

DNA damage progressively accumulates with extended periods in the dry quiescent state (section 1.1.3). Repair of DNA damage in the embryo of is known to occur before completion of germination. Indeed repair is initiated in very early imbibition, detected as high levels of *de novo* DNA synthesis not related to DNA replication (Osborne et al., 1984). The first study to identify a molecular link between DNA repair processes in germination showed that the *LIG4* and *LIG6* are required for vigorous seed germination by facilitating repair of DSBs before germination (Waterworth et al., 2010). Work presented in the previous chapter has shown that *KU70* and *KU80*, involved in NHEJ, also repair facilitate rapid germination (section 3.2.2.5). DSB repair in eukaryotes occurs via two main pathways, NHEJ and HR. This previous work demonstrating a role of c-NHEJ in seed germination raised the question: does HR activity influence germination performance? In high quality seeds, analysis of germination demonstrated that HR mediated by XRCC2 is not an important component of seed vigour; there was no statistical difference between the MGT of the *xrcc2-1* and wild-type seed lots ($P > 0.05$) [Figure 4-2]. This result suggested that HR activity is not limiting for germination vigour. However in high quality seed lots tested under optimal lab conditions, differences in germination potential can be

difficult to identify. Accelerated ageing/controlled deterioration between seed lots have been developed to identity such differences (Matthews et al., 2012).

4.3.3 *xrcc2-1* mutant lines are hypersensitive to accelerated ageing

Accelerated ageing is one treatment used to determine differences in seed lot germination performances (Matthews et al., 2012). When NHEJ-deficient mutants were subjected to accelerate ageing treatments, the MGT increased and viability decreased. This demonstrated that seeds have a lower germination potential when deficient in this type of DSB repair pathway (section 3.2.2.5). It is interesting to note that whilst *xrcc2-1* seeds showed wild-type levels of viability with up to 4 days accelerated ageing ($P > 0.05$) [Figure 4-3], the MGT was significantly different after just 2 days accelerated ageing ($P = 0.008$) [Figure 4-4]. This result indicated that *XRCC2*-mediated HR was required for seed vigour in low quality seeds. Further characterisation of the sub-pathways of HR and understanding which function in response to DNA damage in seeds would better elucidate the overall roles of HR in seed quality (section 1.5.2). The SSA pathway of HR was found to be responsible for a third of repair events between sequence repeats in *Arabidopsis* (Siebert and Puchta, 2002; Waterworth et al., 2011). The DGU.US recombination reporter locus has been engineered to show recombination activity after a DSB event in the *Arabidopsis* genome: this cassette required SSA to restore active the GUS gene which when expressed could be identified by histochemical staining. When the DGU.US recombination reporter was put into *xrcc2* lines, restoration of the GUS gene was severely reduced, demonstrating *XRCC2* was an important factor for SSA activity (Serra et al., 2013). This find suggests that the importance of *XRCC2* in seed vigour in aged seeds displayed in this studied, may be due to its role in the SSA pathway and that conservative repair mechanisms was important in such circumstances.

Phenotypic analysis of irradiated *rad51*-deficient maize kernels showed germination was delayed severely relative wild-type lines with increased numbers of abnormal seedlings. This indicated a role for HR in repair events

during seed imbibition and germination (Li et al., 2007), although potentially irradiation and seed ageing would generate a different spectrum of DNA damage which required different repair mechanisms.

Pre-genomic era work established a correlation between the accumulation of DSBs after seed ageing and seed quality: in high quality seeds levels of DSB DNA damage is much lower (Dourado and Roberts, 1984). Here, germination performance has shown that *XRCC2*-mediated HR was not required for DNA repair, presumably as the c-NHEJ machinery was responsible for repairing the majority of DSBs in higher plants (section 1.5). This also agreed with the phenotypic analysis of mutant alleles from different c-NHEJ and HR-deficient mutants when subjected to genotoxic stress. Mutants deficient in c-NHEJ repair factors such as *KU70* and *KU80* are hypersensitive to the radiomimetic compound bleomycin (Tamura et al., 2002), whilst mutants deficient in HR-factors only show small but significant sensitivity to bleomycin (Tambini et al., 2010), in agreement with the proposed order of DSB repair kinetics in *Arabidopsis* (Charbonnel et al., 2011),

DNA damage results in a wide spectrum of DNA lesions which form substrates for different repair pathways and which can differentially interfere with the essential processes of DNA transcription, replication and mitosis. HR may be required to repair specific substrates, which were present in seeds having undergone accelerated ageing but not high quality seeds. Whilst the majority of S-phase DNA replication synthesis occurs concurrently with radicle emergence and completion of germination (section 1.1.3), work in *Arabidopsis* seeds has shown that replication inhibitors increase MGT (Masubelele et al., 2005). Another HR factor involved in the SSA repair in *Arabidopsis* is *BRCA2* (Seeliger et al., 2012). *BRCA2* in mammals was highly expressed in S-phase and was recruited to stalled replication forks. Functional analysis of *BRCA2* is consistent with a role in recombination repair associated with normal replication (Scully and Livingston, 2000). It is possible that replication blocks caused by DNA damage acquired during accelerated ageing require HR-mediated repair, leading to the MGT increase observed. The majority of replicative DNA

synthesis in *Arabidopsis* occurs at or around radicle emergence (Barrôco et al., 2005; Masubelele et al., 2005). If HR had a role in repair during replication, this would mean a role in DNA repair during seed germination.

The function of HR in the germinating seed should be further investigated. It may be that HR plays a rare but important role in preservation of genome integrity in the germinating seed. Preservation of genomic integrity is of particular importance to meristem cells in order to minimise transmission of mutations in the plant germline, which is established relatively late in the plant development cycle. The embryonic root and shoot apical meristems represent the origin of the new plant (Bewley et al., 2013). Work in *Arabidopsis* root meristems has shown the repair of DSBs is tightly regulated and monitored. Plants with decreased DSB repair capacity show increased programmed cell death events in the meristems in response to DSB-inducing genotoxic stress (Fulcher and Sablowski, 2009).

HR is known to be up-regulated in meristematic cells, whilst meristem cells initiate PCD at lower levels of DNA damage than surrounding tissue (Fulcher and Sablowski, 2009; Waterworth et al., 2011). It would have been interesting to investigate the temporal and spatial localisation of HR transcripts in germinating seeds. Although HR events are rare relative to NHEJ, it is therefore plausible that HR repair of DSBs is particularly crucial in the embryonic meristems.

5. Characterisation of the roles of Nucleotide excision repair and Base excision repair in *Arabidopsis* seed germination

5.1 Introduction

Both base excision repair (BER) and nucleotide excision repair (NER) are involved in the repair of damage that has occurred to one of the two strands of the DNA helix. Whilst these pathways share similar mechanistic steps, including detection of damage, excision around the area of damage and synthesis of new DNA to replace excised bases, the proteins orchestrating these steps differ between the two pathways (sections 1.4.3 and 1.4.4).

To determine if BER and or NER have any role in seed vigour and longevity, core-components of these pathways were selected for study using the same criteria outlined in the previous results chapters. The BER gene *APURINIC ENDONUCLEASE-REDOX PROTEIN* (*ARP*) and NER gene, *EXCISION REPAIR CROSS COMPLEMENTATION GROUP 1* (*ERCC1*) were chosen for study. Mutant lines were then selected that have been previously characterised in *Arabidopsis* and shown to have specific roles in the corresponding repair pathways, displaying DNA damage hypersensitivity so as not to display total redundancy with other genes (section 5.1.1 and 5.1.2). These lines have been previously classified as *arp-1* and *ercc-1* respectively.

5.1.1 ARP and base excision repair

ARP was identified in *Arabidopsis* as one of three genes (including *APE1L* and *APE2*) that shows homology to abasic endonuclease genes in animals, yeast and bacteria. The *Arabidopsis* gene *ARP* was first identified as an analogue of the human REDOX EFFECTOR FACTOR 1/AP ENDONUCLEASE 1 (REF-1/APE1) often shortened to APE1. This endonuclease contains a conserved cysteine residues key for REF/ARE1 redox activity (Babiychuk et al., 1994). The human APE1 was originally isolated as a DNA repair enzyme and later found to have a redox activity. It was found to be responsible for reducing the transcription factors FOS and JUN which enhances DNA-binding activity of both proteins (Xanthoudakis and Curran, 1992, 1996). In *Arabidopsis, ARP* has been found to have this same dual activity *in vitro,* demonstrating both class II endonuclease

activity and redox activity which stimulates the DNA-binding activity of recombinant human transcription factors FOS and JUN (Babiychuk et al., 1994).

Under ideal growth conditions, single mutant lines deficient in any one of the three *Arabidopsis* AP endonucleases do not show any apparent differences from wild-type in growth rate, growth habit, and fertility (Murphy et al., 2009). When *APE1L* and *APE2* are knocked-out concurrently, a lethal phenotype is observed with abortion of developing embryos. Mutant *arp* plants show no such deleterious phenotype even in combination with either *ape1* or *ape2* (Murphy et al., 2009). Work *in vitro* has shown that ARP has the major AP endonuclease activity in *Arabidopsis. arp*-deficient cell extracts lack detectable BER activity on either the uracil- or the synthetic AP site analogue tetrahydrofuran (THF)-containing DNA substrates. This is restored on addition of the human APE1 protein demonstrating APE1L and APE2 cannot redundantly compensate for ARP function in cell extracts (Córdoba-Cañero et al., 2011). Further studies have shown ARP is directly required for 8-oxoG repair, processing the 3′-blocking ends generated by FPG and OGG1 *in vitro* (Córdoba-Cañero et al., 2014).

Whilst *arp-1* plants show no phenotypic difference to wild-type under ideal growth conditions, these lines are hypersensitive to 5-fluorouracil (5-FU), a uracil analogue that favours mis-incorporation of dUMP into DNA. This demonstrates that ARP is an important factor in plant survival and growth under genotoxic stress (Córdoba-Cañero et al., 2011). In the course of the studies presented here, mutant *arp* seeds were reported to display decreased longevity and have increased sensitivity seed ageing by controlled deterioration testing (CDT) (Córdoba-Cañero et al., 2014).

5.1.2 ERCC1 and nucleotide excision repair

ERCC1 encodes a protein critical for stabilising and enhancing the functionality of the endonuclease XPF required for NER (section 1.4.4). The ERCC1/XPF complex creates a structure-specific endonuclease, initially identified for its essential role in nucleotide excision repair (NER) in budding yeast (Davies et

al., 1995). The endonuclease activity specifically recognises double- to single-strand transitions in DNA, incising the 5′–3′ single-strand just after the junction. The protein was first identified in plants in *Lilium longiflorum* (common name, Easter lily) as a homologue to the DNA excision repair proteins human ERCC1 and yeast RAD10. The protein was found to responsible for reduced sensitivity of the cross-linking agent mitomycin C (MMC) in ERCC1-deficient Chinese hamster ovary (CHO) cells (Xu et al., 1998).

Arabidopsis ercc1-deficient plants show hypersensitivity to different DNA damaging agents. UV-B and UV-C treated mutant seedlings have stunted root growth and also display reduced growth in response to methyl methanesulfonate (MMS) and MMC treatment (MMS is an alkylating agent that adds methyl groups to a variety of sites). This shows ERCC1 is important to repair of a wide range of different DNA damage products, as UV creates pyrimidine dimers, (Hays, 2002; Dubest et al., 2004). The gamma-plantlet phenotype is observed after 100 Gy of γ-radiation to *ercc1*-deficient seeds, in which mutants germinate normally and produce healthy seedlings with well-expanded cotyledons, but undergo transient (7 days) late S/G2 arrest in the meristem cells before going on produce true leaves (Hefner et al., 2003; Hefner et al., 2006). Whilst the nature of the arrest-inducing lesion in *ercc1* apical meristem cells is unknown, the late S/G2 associations suggests the damage is not detected until S-phase is underway, possibly a lesion that blocks replication.

ERCC1 has role in SSA homologous recombination in extra-chromosomal recombination assays, where it may act to remove overhanging non-homologous tails after complementary sequences have been exposed and can anneal to each other (Dubest et al., 2004). ERCC1 is also involved in telomere homeostasis in *Arabidopsis*, working in complex with XFP to protect short telomeres from homologous recombination. Whilst *ercc1* mutants develop normally and show wild-type telomere length, *Arabidopsis* plants that are also *tert*-deficient display much earlier onset of chromosomal instability. This telomere instability is associated with the presence of dicentric chromosome

bridges; cytological studies show visible extra-chromosomal DNA fragments in mitotic anaphase rather than a general acceleration of telomeric repeat loss (Vannier et al., 2009).

5.1.3 Aims

Recent work has shown components of the NHEJ repair pathway to be important in seed vigour. Work in chapter 3 showed *ku70* and *ku80* deficient seeds are hypersensitive to accelerated ageing treatment. Furthermore mutant lines deficient in *lig4* and *lig6* show a marked delay in germination and loss of viability. The work reported to date has demonstrated a role for DSB repair factors in seed quality, but it leads to the question: Do any other repair pathways have a similar importance in seed vigour? Through analysis of the germination performance of seed deficient in different repair pathways, other factors which determine seed quality can potentially be identified. Some SSB repair factors involved in BER have now been associated with seed longevity. Hypersensitivity to ageing treatments in *Arabidopsis* seeds deficient in the BER factor *arp* and *Arabidopsis* seeds overexpressing another BER factor OGG1 demonstrate resistance to ageing treatments (Chen et al., 2012; Córdoba-Cañero et al., 2014). No direct evidence has been reported linking the NER repair pathway with germination performance. Here the germination performance analysis and comparison of mutants deficient in either core NER or BER factors demonstrate that seed longevity is associated with active base excision repair but not nucleotide excision repair.

5.2 Results

5.2.1 Isolation of *arp*- and *ercc1*-deficient lines

To determine the relative roles that BER and NER might play in seed vigour, viability ARP (AT2G41460) and ERCC1 (AT3G05210) were chosen as their roles in DNA repair (BER and NER respectively), which have been well characterised in *Arabidopsis* (sections 5.1.1 and 5.1.2). Previously characterised T-DNA insertion lines were identified using the SIGNAL flanking sequence tag database (http://signal.salk.edu/cgi-bin/tdnaexpress) and ordered through the Nottingham *Arabidopsis* Stock Centre (NASC, http://*Arabidopsis*.info) (Scholl et al., 2000) (section 2.3). Both mutant lines come from the SALK T-DNA mutant collection (Alonso et al., 2003). The mutant line for *ARP* was designated *arp-1* (SALK_021478) (Córdoba-Cañero et al., 2011) and *ERCC1* was designated *ercc1-1* (SALK_033397) (Dubest et al., 2004) [Figure 5-1 A, 5-2 A]. These mutants are the result of the *Agrobacterium tumefaciens*-mediated integration of the T-DNA region of the pROK2 vector. PCR was performed on the lines obtained from the stock centre to confirm the presence of the T-DNA insertion [Figure 5-1 B, 5-2 B]. The presence of the T-DNA allele was identified by the amplification of a PCR product in a reaction using primer corresponding to the LB region of the T-DNA insert and gene specific primers for *arp-1* and *ercc1-1* as shown in [Figure 5-1 B, 5-2 B]. Wild-type alleles were detected using a pair of primers designed to the *ARP* and *ERCC1* genomic sequence that spanned the region of T-DNA insertion and due to the large size of the T-DNA (*c.* 4 500 bp) and PCR cycle parameters, these primers were unable to amplify a PCR product in the presence of the T-DNA insertion.

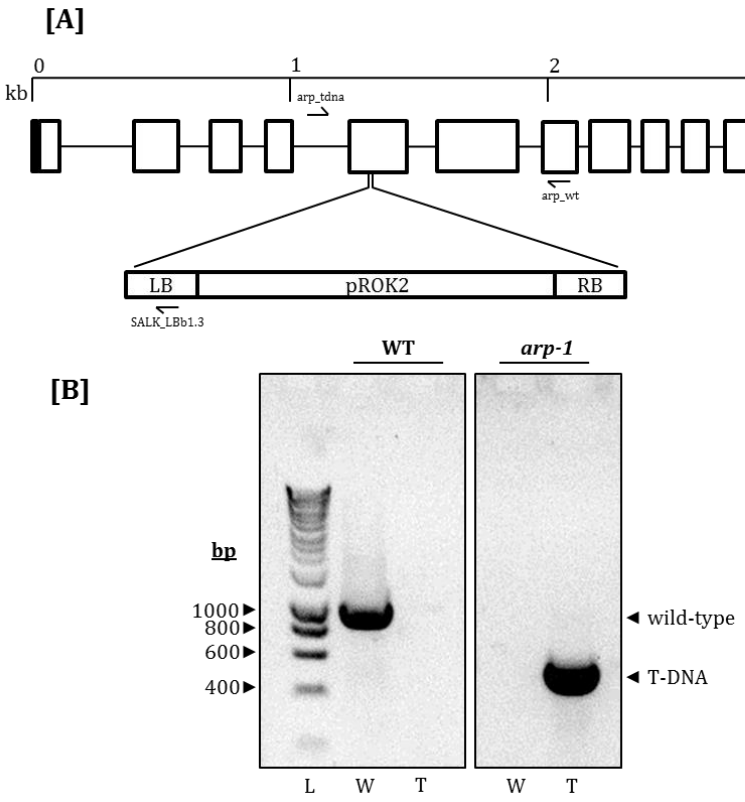


Figure 5-1 Isolation of *arp-1* T-DNA insertion mutant.

[A] Schematic of the *Arabidopsis ARP* gene. Exons are denoted by boxes, with filled boxes representing UTR, empty boxes representing the coded regions and introns represented by a line. The position of the T-DNA insertion is indicated in the 5th exon. The location of the primers used for genotyping *arp-1* are shown in black, with the T-DNA allele identified using primers arp_tdna and SALK_LBb1.3 and the wild-type allele identified using primers arp_tdna and arp_wt [Table 2.2].

[B] Left Border PCR of *arp-1* T-DNA insertion. Genotyping to identify wild-type (WT) and homozygous *arp* plants for the T-DNA allele. The PCR used primers shown in Figure 3-1. The ladder used was Hyperladder 1 kb plus (lane L). Wild-type plants resulted in only a wild-type band (lane W) and homozygous plants had only the T-DNA band (lane T). PCR amplification of genomic DNA (section 2.7) was performed using DreamTaq PCR reaction mastermix (section 2.7.1) with the PCR program Taq55TD (section 2.7.2) in 20 µl.

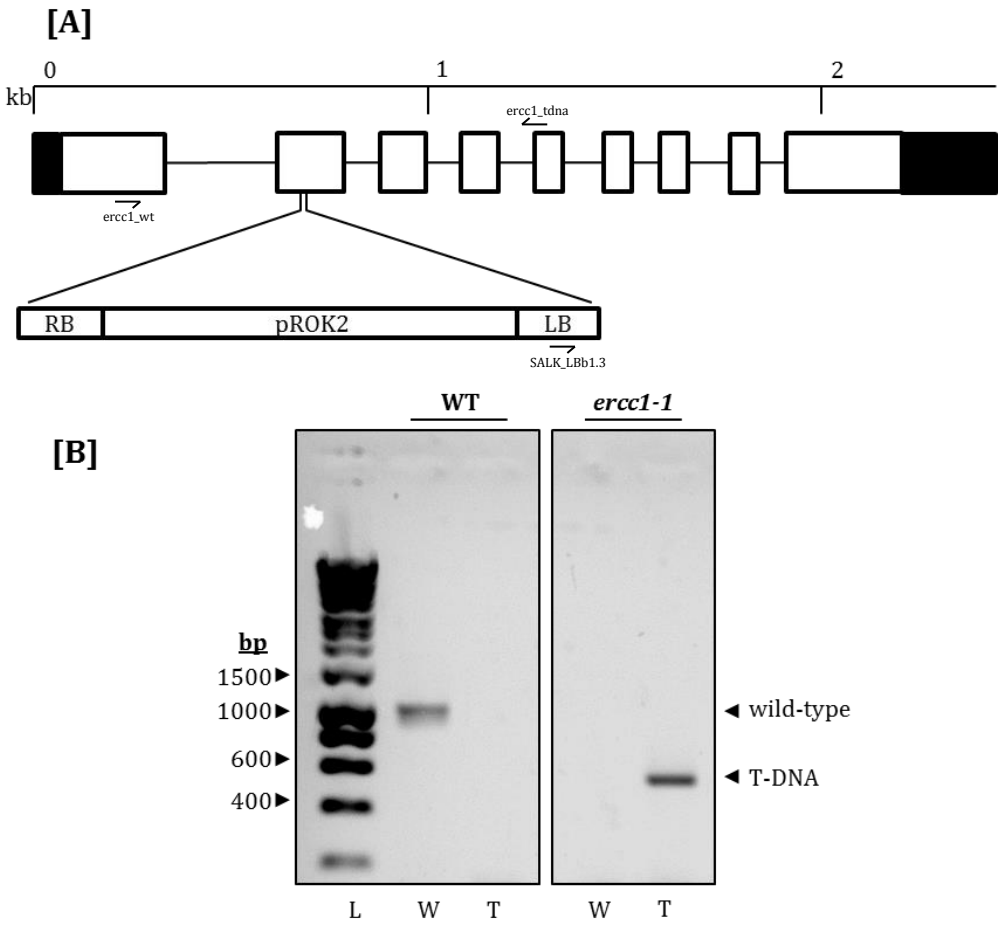


Figure 5-2 Isolation of *ercc1-1* T-DNA insertion mutant.

[A] Schematic of the *Arabidopsis ERCC1* gene. Exons are denoted by boxes, with filled boxes representing UTR, empty boxes representing the coded regions and introns represented by a line. The position of the T-DNA insertion is indicated in the 2nd exon. The location of the primers used for genotyping *ercc1-1* are shown, with the T-DNA allele identified using primers ercc1_tdna and SALK_LBb1.3 and the wild-type allele identified using primers ercc1_tdna and ercc1_wt [Table 2.2].

[B] Left Border PCR of *arp-1* T-DNA insertion. Genotyping to identify wild-type (WT) and homozygous *ercc1-1* plants for the T-DNA allele. The PCR used primers shown in [Figure 5-2 A]. The ladder used was Hyperladder 1 kb plus (lane L). Wild-type plants resulted in only a wild-type band (lane W) and homozygous plants had only the T-DNA band (lane T). PCR amplification of genomic DNA (section 2.7) was performed using DreamTaq PCR reaction mastermix (section 2.7.1) with the PCR program Taq55TD (section 2.7.2) in 20 µl.

5.2.2 Sensitivity of *arp-1* and *ercc1-1* to X-ray

Here, wild-type, *arp-1* and *ercc1-1* seed were treated with X-rays as in chapter 3. Seed lots were surface sterilised in 70% ethanol for 5 min, and resuspended in dsH_2O before IR treatment, (75 Gy), delivered using a 320 kV X-ray irradiation system (NDT Equipment Services) at a rate of 1 Gy $minute^{-1}$. Seeds were plated individually in one row on ½ Murashige and Skoog agar before being stratified at 4 °C for 48 hours (section 2.4). The plates were then transferred to a growth chamber and grown vertically down the surface of the agar (section 2.5.4). The primary roots were measured 3, 5, and 7 days after plates had been transferred to the growth chamber and the mean root length was calculated [Figure 5-3 A].

Neither mutant displayed a different phenotype in root growth relative to wild-type without X-ray treatment, this agrees with previous characterisation of the two knockout lines (Dubest et al., 2004; Córdoba-Cañero et al., 2011). Hypersensitivity to X-ray treatment is observed in only the *ercc1-1* mutant line; with slower growth phenotype observed when compared to wild-type. [Figure 5-3] shows the statistical significance between each appropriate comparable measured average. After 75 Gy treatment, *ercc1-1* shows much slower root growth than wild-type, using a one-way ANOVA with a Tukey HSD post-hoc test for multiple comparisons is confirmed highly significant ($P < 0.05$).

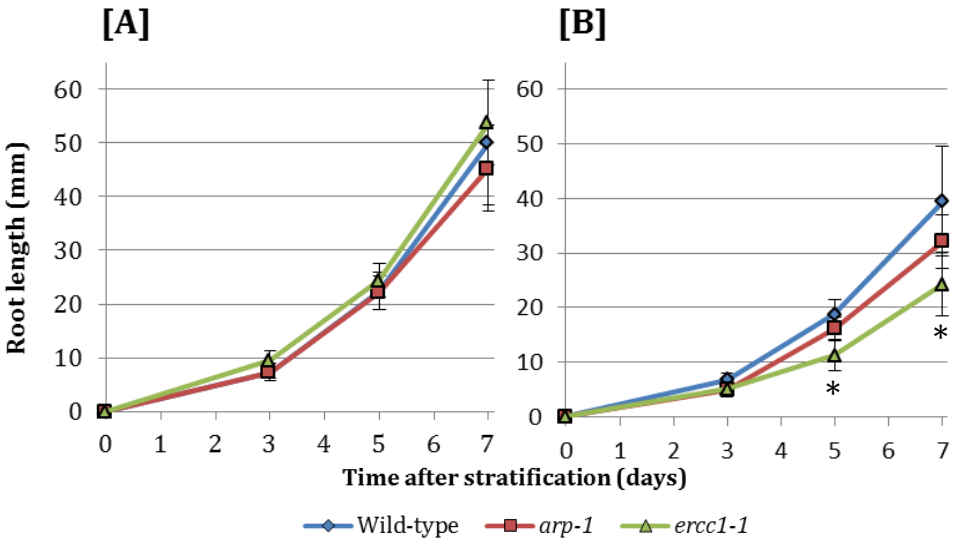


Figure 5-3 Sensitivity of *arp-1* and *ercc1-1* mutants to x-ray.

[A] Mean root length with no x-ray treatment. Wild-type, *arp-1* and *ercc1-1* lines were grown as previously described (section 2.5.4). The mean length of primary roots is shown after 0, 3, 5 and 7 days post imbibition from 1 plate with respect each mutant line (minimum n=7). Error bars show the standard error of the mean (SD). The probability associated with Levene's Test for Equality of Variances at three days (p=0.455), five days (p=0.255) and seven days (p=0.005). A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD test indicated no significant differences (P>0.05) between the means score for 3 days (wild-type; M = 7.37 mm SD = 1.98, *arp-1*; M = 7.50 mm SD = 1.56, *ercc1-1*; M = 9.04 mm SD = 1.99) and 5 days growth (wild-type; M = 22.38 mm SD = 3.71, *arp-1*; M = 22.16 mm SD = 2.94, *ercc1-1*; M = 24.19 mm SD = 5.56). Post hoc comparisons using the Tukey HSD test with the Games-Howell corrected indicated no significant differences (P>0.05) between the growth of wild-type and the mutant lines (wild-type; M = 48.19 mm SD = 13.93, *arp-1*; M = 45.67 mm SD = 7.46, *ercc1-1*; M = 53.57 mm SD = 7.2)

[B] Mean root length with 75 Gy X-ray treatment: Wild-type (n = 11), *arp-1* (n = 8) and *ercc1-1* (n = 11) mutant lines were treated with a 75 Gy dose of x-ray and root growth recorded as previously described (section 2.5.4). The probability associated with Levene's Test for Equality of Variances at 3 days (p=0. 200), 5 days (p=0. 522) and 7 days (p=0.073). A one-way ANOVA was conducted to compare root growth between the 3 genotypes. Post hoc comparisons using the Tukey HSD indicated the means score at 3 days growth (wild-type; M = 6.81 mm SD = 1.03, *arp-1*; M = 4.95 mm SD = 1.46, *ercc1-1*; M = 5.10 mm SD = 0.39) are significantly different in the *ercc1-1* lines (p = 0.01) when compared to wild-type root growth. After 5 days growth (wild-type; M = 18.49 mm SD = 2.73, *arp-1*; M = 16.14 mm SD = 2.76, *ercc1-1*; M = 11.18 mm SD = 2.08) shows a significant difference in *ercc1-1* lines (p < 0.0005), when compared to wild-type root growth. There was no significant difference found between the mean of wild-type and *arp-1* lines (p = 0.129). 7 days growth (wild-type; M = 39.46 mm SD = 9.63, *arp-1*; M = 32.21 mm SD = 5.90, *ercc1-1*; M = 24.32 mm SD = 4.91) shows a significant difference in *ercc1-1* root growth (p<0.0005) when compared to wild-type root growth. There was no significant difference found between the mean of the *arp-1* and *ercc1-1* (p = 0.136). * indicates a P < 0.05 significant difference between wild-type and *ercc1-1* root growth means.

5.2.3 Germination performance of high quality *arp-1* and *ercc1-1* seeds is indistinguishable from wild-type seeds

ercc1-1 displayed sensitivity to X-ray treatment and the knockout lines have been previously characterised as being deficient in the single strand break repair pathway NER (Dubest et al., 2004). Germination assays were next performed on high quality seeds lots (section 2.5.1) in wild-type, *arp-1* and *ercc1-1* background to determine if SSB repair pathways have a role in seed quality. Seeds were plated onto germination paper (Anchor Blue, California) in a 90 mm petri dish with 7.5 ml dH_2O and stratified for 2 days before being moved to a growth chamber (section 2.5). Germination was scored starting 22 hours after seeds were moved to the growth chamber following stratification. The germination performance of *arp-1* was compared to wild-type seeds to determine any delay to germination, similarly the germination of *ercc1-1* was shown [Figure 5-4 A].

The graph [Figure 5-4 B] showed no significant differences ($P > 0.05$ using an unpaired one-way ANOVA and Tukey HSD post-hoc test for multiple comparisons) between MGTs of mutants and wild-type (section 2.5). [Figure 5-4 B] also showed no significant difference in MGT between either *arp-1* and *ercc1-1* and wild-type ($P > 0.05$). This suggests that in high quality seeds BER and NER did not play an important role in seed vigour.

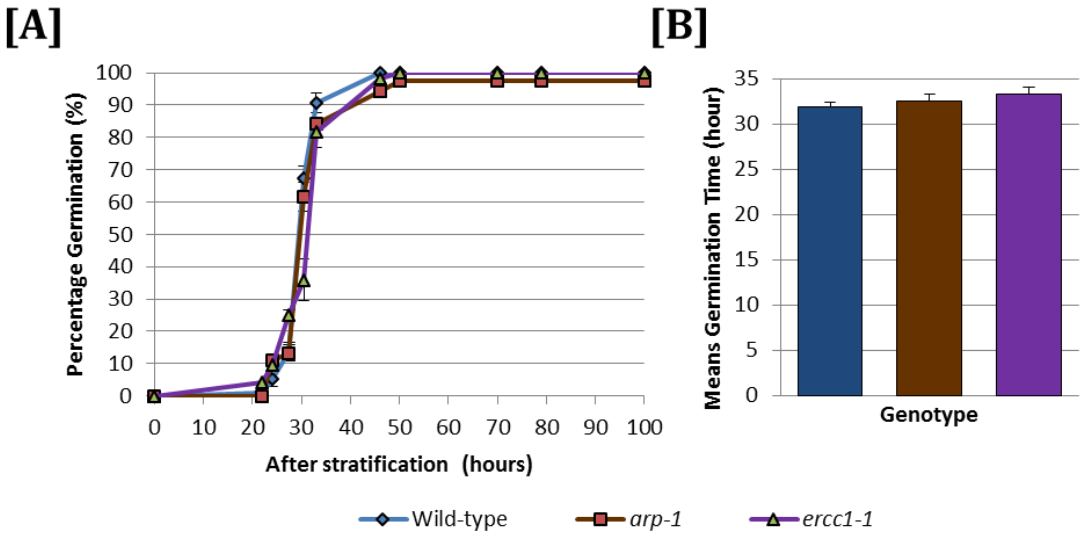


Figure 5-4 Analysis of the germination performance of high quality single strand break repair mutants.

[A] Germination performance of high quality *arp-1* and *ercc1-1* mutant lines. Germination performance of *arp-1* and *ercc1-1* seeds analysed at 23 °C under 16 hour light and 8 hour dark cycles. Seeds were plated on germination paper and stratified at 4 °C for 48 hours before transfer to 23 °C (section 2.5).

[B] *arp-1* and *ercc1-1* mean germination time. Mean germination time (MGT) for physiological germination is presented. Error bars show the standard error around the mean (SEM). Wild-type M = 31.91 hours (SEM = 0.83), *arp-1* M = 32.46 hours (SEM = 0.08), *ercc1-1* M = 33.33 hours (SEM = 0.71). The probability associated with Levene's Test for Equality of Variances (p=0.18), A one-way ANOVA for multiple comparison using the Games-Howell correction was conducted to compare the MGT indicated that there was no significant differences between *arp-1* and *ercc1-1* and wild-type ($P > 0.05$) .

5.2.4 Hypersensitivity to accelerated ageing treatment is observed in *arp-1* lines but *ercc1-1*

The ageing regime chosen was relatively mild, with the seeds only exposed to high humidy (82 % relative humidity) and a temperature of 40 °C for 48 hours. The regime caused no significant loss of viability to wild-type seeds ($P > 0.05$, Students T-Test comparing 2 days aged and untreated wild-type seed lots) [Figure 5-5 A]. In wild-type seeds, ageing resulted in a near 2-fold increase in the MGT compared to that observed in high quality seeds and there a decreased seed quality through a decrease in seed vigour was observed.

Work presented in [Figure 5-5 A] showed the germination performance of 2 days aged *arp-1* and *ercc1-1.* To determine if there was any difference in seed vigour, the MGT was calculated and an unpaired multiple comparison one-way ANOVA was performed [Figure 5-5 B]. The results showed the *arp-1* line had a significantly longer MGT than that of wild-type ($P = 0.001$). This suggested that *ARP* has an important role in protecting and repairing the genome upon imbibition before germination. This result highlighted the potential of BER more widely having an important role in germination. By contrast the *ercc1-1* mutant line displayed no significant increase in MGT when compared with wild-type under the conditions used, consistent with *ercc1-1,* and by extension NER, not having an important role in seed longevity and vigour. This did not rule out the possibility that NER does play some role in seed germination when the seed is exposed to other exogenous stresses.

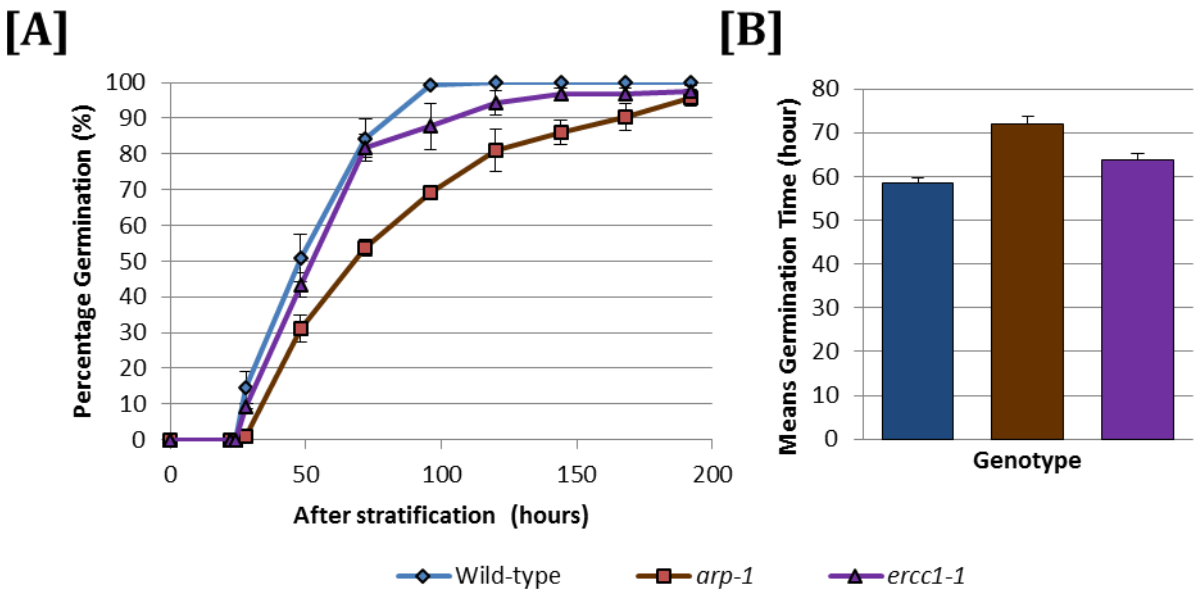


Figure 5-5 Analysis of single strand break germination performance after 2 days accelerated ageing.

[A-B] Germination performance in 2 days accelerated aged *arp-1* and *ercc1-1* mutant seed lines. Germination performance of *arp-1* and *ercc1-1* seeds, analysed at 23 °C under 16 hour light and 8 hour dark cycles. Seeds were plated on germination paper and stratified at 4 °C for 48 hours before transfer to 23 °C (section 2.4)

[C] Mean time to germination of each mutant line. Mean germination time (MGT) for physiological germination is presented. Error bars show the standard error around the mean (SEM). Wild-type M = 58.57 hours (SEM = 1.04), *arp-1* M = 72.10 hours (SEM = 1.55), *ercc1-1* M = 63.86 hours (SEM = 1.37). The probability associated with Levene's Test for Equality of Variances (p=0.70), A one-way ANOVA for multiple comparison using the Games-Howell correction was conducted to compare the MGT indicated that there was no significant differences between *ercc1-1* and wild-type (P = 0.84). Statistical analysis shows a significant difference between wild-type MGT and *arp-1* MGT (P = 0.007).

5.3 Discussion

5.3.1 Molecular analysis of the *arp-1* and *ercc1-1* mutant lines

T-DNA mutant lines were isolated to allow a reverse genetics approach to determine the function of *ARP* and *ERCC1* with regards to germination longevity and vigour. Each mutant line was predicted to have a T-DNA insertion within their respective gene and have been previously characterised to confirm this. Neither T-DNA insertional line produce transcripts or proteins of their respective gene and furthermore the *arp-1* and *ercc1-1* mutant lines have previously been identified and characterised for deficiencies in BER and NER respectively (Dubest et al., 2004; Córdoba-Cañero et al., 2011). PCR-based genotyping was used here to confirm that homozygous lines of these characterised mutant alleles were obtained.

For this study the mutant allele for each gene was chosen as they have been previously well characterised over multiple studies (Dubest et al., 2004; Hefner et al., 2006; Murphy et al., 2009; Vannier et al., 2009; Córdoba-Cañero et al., 2011, 2014) and the aim of this research is to determine any role each pathway rather than gene, may have on seed germination.

5.3.2 Sensitivity of *arp-1* and *ercc1-1* to X-rays

The use of radiation hypersensitivity of plant growth in forward genetics has long been an established route into characterisation of DNA repair genes; indeed it is how *ERCC1* in *Arabidopsis* was first identified (Xu et al., 1998). Root growth assays performed after irradiation can be a strong indicator of such DNA repair roles. The sensitivity of *ercc1* deficient mutants to radiation is well characterised and the results presented here concur with what is previously reported (Hefner et al., 2003; Dubest et al., 2004). Root growth retardation has not been reported in *arp-1* deficient plants after radiation and analysis here

showed no hypersensitivity: however it has been reported that these mutants are sensitive to other DNA damaging agents such as 5-fluorouracil (Córdoba-Cañero et al., 2011).

Intermediates of both NER and BER are regions of single stranded DNA. If either repair pathway is impeded through, for example the loss of one of the constituent parts, this can lead to higher incidence of DSB events at these points of ssDNA (Mladenov and Iliakis, 2011). This potentially causes retardation of root growth in NER mutants due to an increased persistence of DSBs causing a pause in cell cycle progression with even the loss of some stem cells through PCD.

5.3.3 Germination vigour is not affected by the loss of *ARP* or *ERCC1* in high quality seeds

Repair of DNA damage in the embryo of *Arabidopsis* seeds is known to occur before germination was completed. Indeed repair is initiated in very early imbibition, detected as high levels of *de novo* DNA synthesis (Osborne et al., 1984). The studies in Chapter 3 have identified that DSB repair genes are important determinants of seed viability, vigour and longevity**.** The work presented here extends this understanding of seed quality by mutant analysis of BER and NER factors and their influences on seed vigour. Using the *arp-1* and *ercc1-1* mutant alleles to investigate roles of BER and NER respectively in seed quality, germination assays were performed on high quality seeds (section 2.5.1). These two mutant lines displayed the same germination characteristics as wild-type under the conditions used consistent with no important role in germination for the BER and NER repair pathways here.

5.3.4 *arp-1* but not *ercc1-1* seeds demonstrate increased susceptibility to accelerated ageing.

To further elucidate any role *ARP* and *ERCC1* might play in germination, accelerated ageing was used. This exacerbates any differences the knock-out mutant might have in comparison to wild-type.

Interestingly after ageing, the BER deficient seeds (*arp-1*) showed a significant increase in MGT ($P < 0.01$), and therefore decreased seed vigour. Conversely NER deficient seeds (*ercc1-1)* did not. The importance of BER to seed vigour in aged seeds over NER could be explained through the types of DNA damaged incurred during the ageing process and imbibition. Oxidative damage is associated with seed desiccation and storage (Dandoy et al., 1987; Bray and West, 2005). Among all the DNA lesions induced by ROS, 7,8-dihydro-8-oxoguanine (8-oxoG) is predominant, resulting from of ROS-induced hydroxylation of the C-8 position of guanine (Kasai and Nishimura, 1984), which is shown to significantly increase in seeds during ageing and imbibition (Chen et al., 2012). BER is the major repair pathway to remove 8-oxoG damage (Kasai and Nishimura, 1984), consistent with the importance of the BER factor *ARP* in seed vigour demonstrated here. Recent work using a different seed ageing technique known as controlled deterioration (section 1.1.4.2) was reported and showed a similar decrease in vigour to the data presented here (Córdoba-Cañero et al., 2014). These results agreed with this finding, but this work further showed the potential importance of BER in seed longevity over NER. Expression of the BER-associated genes *MtTdp1α* and *MtTdp1β* in *Medicago truncatula* is up-regulated in seeds (Macovei et al., 2010) and work in *Arabidopsis* has demonstrated that over-expression of another BER component, OGG1, enhanced seed longevity and abiotic stress tolerance (Chen et al., 2012). This ageing regime resulted in delayed radicle emergence in wild-type seed, with MGT increased from 32 hours in high quality seed to 58 hours in aged seeds [Figure 5-4 B and 5-5 B]. No decrease in viability was observed and remained at *c.* 100 % [Figure 5-5 A]. Delayed germination of aged seeds may result from the operation of checkpoints prior to the commencement of DNA replication that control progression through germination in response to the accumulation of genomic damage. The MGT of unaged and aged *ercc1* seeds were not significantly different to wild-type and the total viability remained close to 100%, showing NER repair pathway does not have an important determining role in seed vigour, viability or storability under the conditions tested. However this did not rule out the possibility that NER does play some

role in seed germination but required under different environmental stresses not determined here.

This work demonstrated the importance of *ARP*-mediated BER but not NER to seed vigour after seed ageing. Interesting, *arp-1* did not show the same levels of hypersensitivity to accelerated ageing as the *ku70-1* and *ku80-3* mutants [Figure 3-11], where significant decreases in viability were observed. Under the conditions tested here, this work demonstrated that whilst BER is important to seed quality is it less so than NHEJ.

6. Characterisation of the plant specific DNA ligase 6

6.1 Introduction

DNA ligases are broadly divided into two groups based on the utilisation of different co-factors, either NAD^+ or ATP (Shuman, 2009). NAD+-dependent DNA ligases are mostly observed in eubacteria whereas ATP-dependent DNA ligases are utilised primarily by eukaryotes. Several ATP-dependent DNA ligases are found across the eukaryote domain which have specific roles in cellular DNA metabolism (Martin and MacNeill, 2002).

6.1.1 DNA Ligases

Four DNA ligases have been characterised in eukaryotes, DNA LIGASE 1, 3, 4 and 6 (LIG1, LIG3, LIG4 and LIG6 respectively). *LIG1* and *LIG4* are conserved across all eukaryotes (Shuman, 2009). *LIG3* homologues are found across eukaryotes including in human, fruit fly, sea anemone and slime mould but are absent in some eukaryotic groups including plants (Simsek and Jasin, 2011). *LIG6* is structurally distinct to other DNA ligases and was first identified in *Arabidopsis* and rice (*Oryza sativa*) by sequence identity to *LIG1* and has only been observed in higher plants (Bonatto et al., 2005; Waterworth et al., 2010). DNA LIGASE 2 and DNA LIGASE 5 were originally characterised in mammalian cells and have biochemical ligase activities but no known function and do not represent additional LIG genes (Tomkinson and Mackey, 1998): DNA LIGASE 2 might result from alternative splicing of *LIG3* transcripts and DNA LIGASE 5 is thought to be derived from one of the known mammalian *LIG* genes (Shuman, 2009; Simsek and Jasin, 2011).

In mammals, *LIG1* is an essential gene that encodes the major cellular DNA ligase activity that joins Okazaki fragments as part of the DNA replication complex (Barnes et al., 1992). *LIG1* homologous have been identified across the eukaryotic spectra with well characterised examples in the yeast, *Saccharomyces cerevisiae* (Barnes et al., 1992) and in *Arabidopsis* (Taylor et al., 1998). *LIG1* homologues have important roles in SSB repair in eukaryotes with additional DSB repair roles characterised in animals (Liang et al., 2008) and plants (Waterworth et al., 2009). The *Arabidopsis* plant cell contains three

genomes, those of the nucleus, plastids and mitochondria (*Arabidopsis* Genome Initiative, 2000). The *Arabidopsis LIG1* gene encodes two isoforms shown by GFP fusions, one targeted to the nucleus and the other the mitochondrion. These isoforms arise from alternative translation start sites in a single mRNA transcript, producing LIG1 protein isoforms either with or without a mitochondrial targeting pre-sequence (Sunderland et al., 2006). No reported evidence could be found *in planta* demonstrating LIG1 is targeted to the chloroplast (Sunderland et al., 2006). However *LIG1* transcripts do contain a theoretical chloroplasts targeting sequence (Sunderland et al., 2006) and double *lig6 lig4* mutants are viable and green (Waterworth et al., 2010) suggesting LIG1 may be responsible for chloroplast genome maintenance and replication but remain inconclusive.

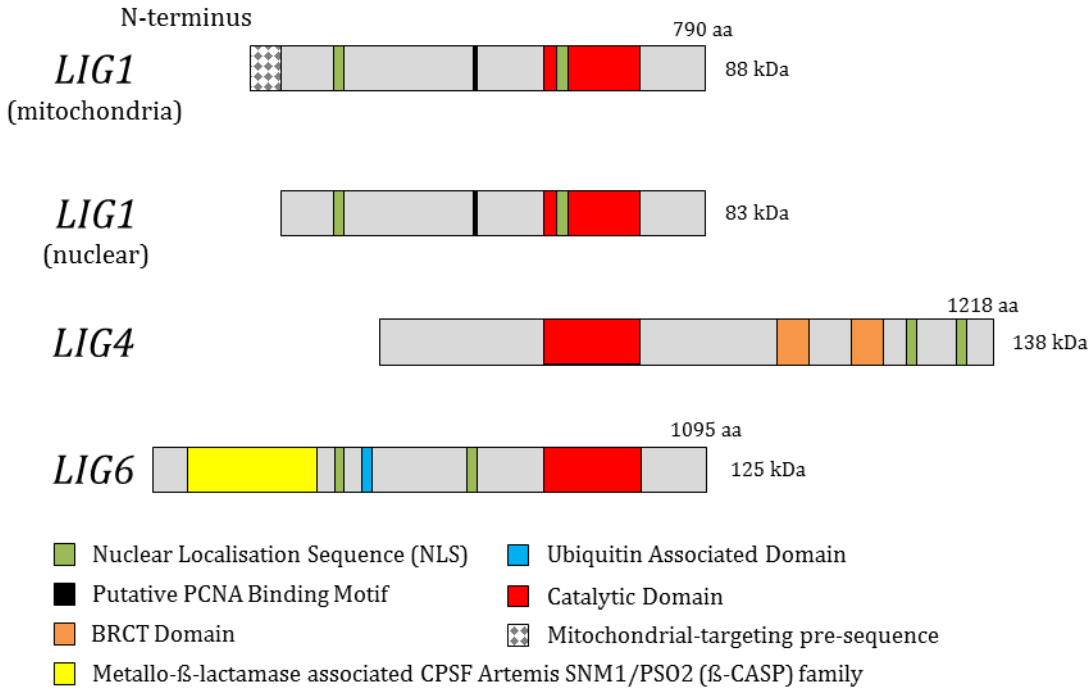


Figure 6-1 Schematic of Ligase proteins found in *Arabidopsis*

6.1.2 Elucidating the function of LIG6 in higher plants

Characterisation in *Arabidopsis* seeds showed that LIG6 is required for rapid seed germination, seedling establishment and is a determinant of seed quality and longevity (Waterworth et al., 2010). Seedlings deficient in *LIG6* show no increased growth sensitivity to a wide range of DNA damaging agents including: UV-C, methyl methanesulfonate (MMS), mitomycin C (MMC), menadione and bleomycin (Waterworth et al., 2010), unlike the hypersensitivity observed in other *Arabidopsis* knock-out lines of DSB repair factors including *lig4* lines (West et al., 2000). Only with exposure to a high dose of X-rays is significantly increased growth retardation observed relative to wild type plants (Waterworth et al., 2010), indicative of a role in DSB repair.

The mechanism by which *LIG6* interacts with the different DNA repair pathways has yet to be elucidated. Work in *Arabidopsis* has shown a function for *LIG6* in the repair of the hairpin-ended double strand breaks (DSBs) generated during excision of the maize Ac element. These results suggest *LIG6* is a functional homologue of ARTEMIS (section 1.5.3.1), where *LIG6* binds to a hairpin structure and either nicks/facilitates hairpin nicking close to their apex. In the absence of a functional LIG6, other nucleases may open the hairpin structures, but tend to do so further from the hairpin apex (Huefner et al., 2011). A non-essential role for *LIG6* has also been observed in *Agrobacterium*-mediated T-DNA integration into the *Arabidopsis* genome, where the presence of LIG6 limits T-DNA integration in root transformation (Park et al., 2015).

6.1.3 Aims

Recombination activities in a plant cell can be observed using an extra-chromosomal recombination (ECR) assay. Two plasmids containing the respective 3´and 5´ends of GFP and sharing some region of sequence homology are transfected into cells. Recombination between the two plasmids is measured by the activation of a reporter gene. Here, an *in planta* ECR assay has been developed utilising *GFP* as the reporter gene to determine any roles *LIG4* and *LIG6* have in ECR. Performing this analysis on knockout mutants of *lig4*,

lig6 and *lig6 lig4*, ratios of GFP:dsRED fluorescence gave quantifiable measures of the ECR rate in each genotype. Any statistically significant changes in this ratio in the mutant protoplasts when compared to wild-type would implicate that gene in the control of extra-chromosomal recombination activity.

6.2 Results

6.2.1 *LIG6* promotes extra-chromosomal recombination events in *Arabidopsis* protoplast cells whilst *LIG4* inhibits ECR.

To investigate the potential roles of LIG4 and LIG6 in ECR, a modified recombination assay from those of the 1990's was used. Instead of GUS, two fluorescent proteins were used to analyse recombination events; GFP and dsRED. dsRED was used to quantify plasmid uptake into protoplasts whilst two plasmids, each containing a different deletion of the GFP gene with an overlapping middle sequence was used to assay recombination. Only if a recombination event had occurred between the two GFP plasmids would a functioning fluorescent GFP protein be expressed (section 2.11). Fluorescence was quantified using confocal microscopy and controls were performed to ensure no detectable crossover in simultaneous excitation can be established when visualising any dsRED or GFP associated fluorescence [Figure6-2] (Drexler et al., 2004).

This assay was used to evaluate the relative rates of recombination in protoplasts deficient in *LIG4, LIG6* or both *LIG4* and *LIG6* in comparison to wild-type. Measurements were determined by taking the ratio of fluorescence (GFP:dsRED): the higher the value, the greater the fluorescence of GFP is in comparison to dsRED and therefore increased incidence of recombination. This assumes that there was no bias in the plasmid transfection between by each genotype. A significant difference between the ratios observed in the *lig4-5, lig6-1*, *lig6-1 lig4-5* and wild-type protoplasts would implicate a role for these gene(s) in ECR.

To test levels of co-transformation, wild-type protoplasts were transformed with plasmid containing the full length gene for GFP and a plasmid containing the gene for dsRED. Levels of co-transformation were ascertained by comparing the number of transformed protoplasts fluorescing both red and

green to those fluorescing only one colour. Levels of co-transformation were at 100% and overall transformation was c.40 % [Figure 6-2]. It was hypothesised that if a protoplast had taken up the plasmid containing the gene for dsRED, the same amount of plasmid containing the gene for GFP should be co-transformed into the cell.

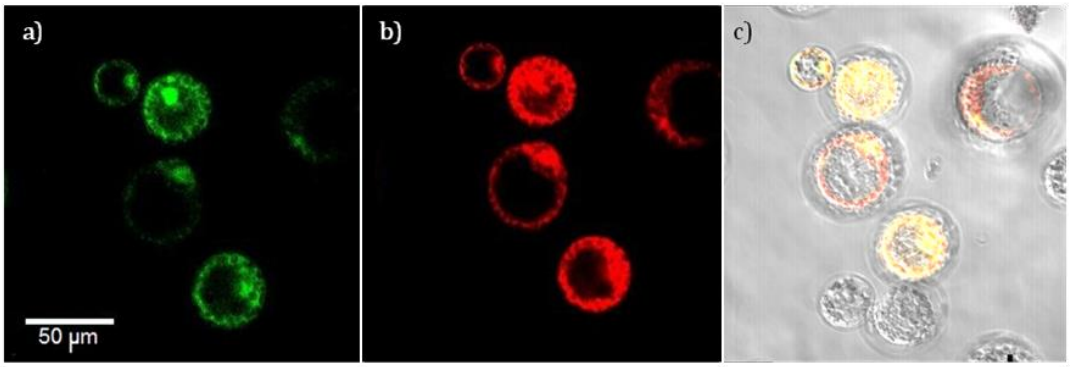


Figure 6-2 Efficiency of protoplast transformation

Showing 100% co-transformation **[A]** imaged for GFP fluorescence (488 nm wavelength light) **[B]** imaged for dsRED fluorescence (543 nm light wavelength) and **[C]** imaged for both GFP and dsRED as well as illumination to show all protoplasts.

The GFP:dsRED ratio of transformed wild-type *Arabidopsis* protoplasts was measured to provide a control for the relative fluorescence. A variation in values was observed ranging from 0.028435 to 4.483924 with an average of 1.192819. The Anderson-Darling test was used to determine whether the data was normally distributed: -

$$AD = -N\frac{2i-1}{N}(\ln(F(Y_i)) + \ln(1 - F(Y_{N+1-i})))$$

Equation 6-1 Anderson-Darling test for normal distribution

N is the number of data F is the cumulative distribution factor and Y is the succession of ordered values. This test assumes normality, and is looking for sufficient evidence to reject normality. The likelihood of normality is expressed as a P-value, with normality accepted with a value P >0.05 i.e. within a 95% confidence interval.

The P-value for this data was calculated to be 0.0178224, indicating that the data is not normally distributed [Figure 6-4]. The ratiometric data was transformed by taking the log of each value. Applying the Anderson-Darling test to test whether the transformed data was normally distributed gave a P-value = 0.592625, falling well within the confidence range.

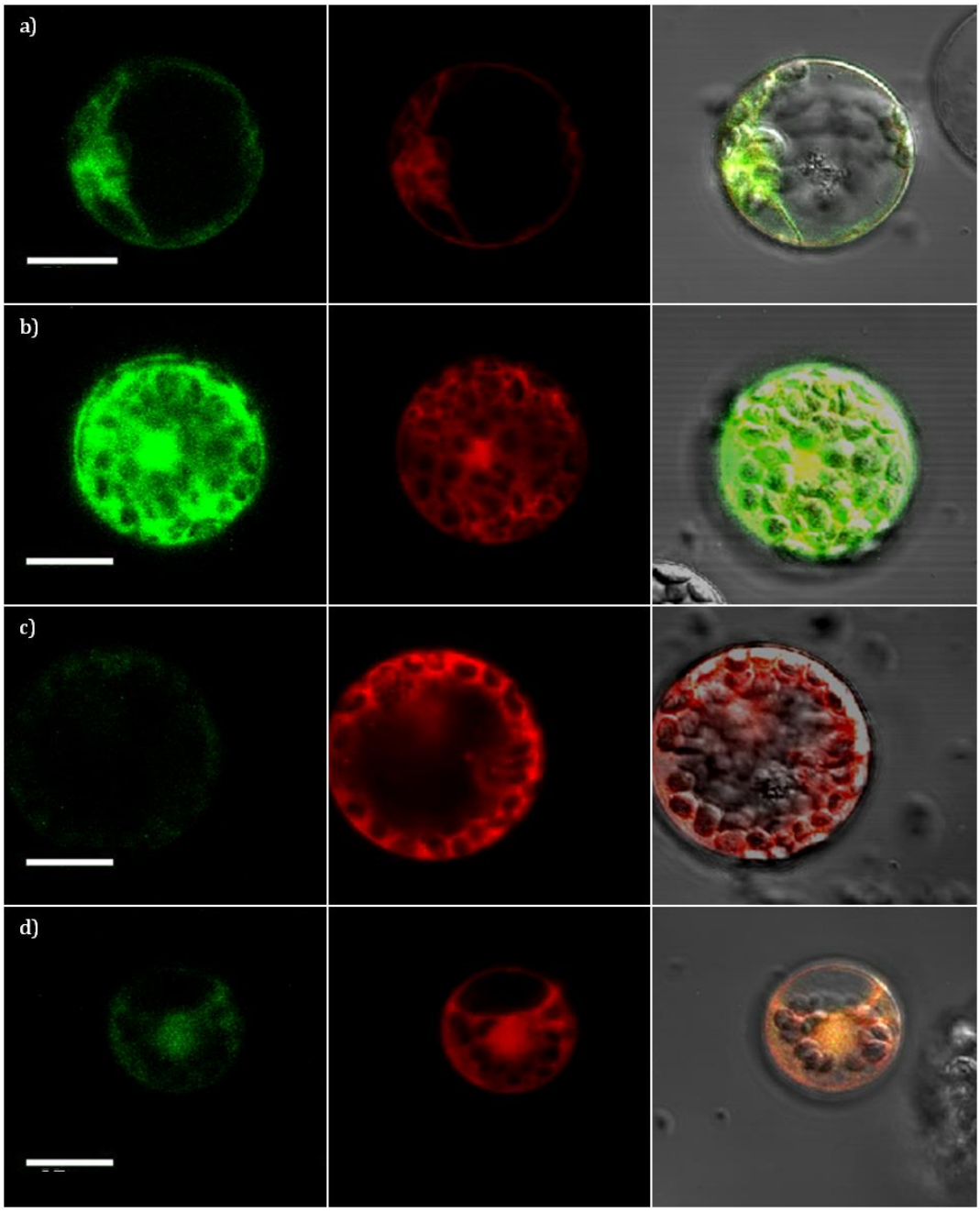


Figure 6-3 Extra-chromosomal assay showing GFP and dsRED fluorescence

Transformed protoplasts with 5'END DEL GFP, 3'END DEL GFP and dsRED a) wild-type, b) *lig4-5* c) *lig6-1* d) *lig6-1 lig4-5.* (Scale bars: 25 µm). For the ECR assay, relative fluorescence of recombined GFP to dsRED was measured in wild-type, *lig4-5*, *lig6-1* and the *lig6-1 lig4-5* double mutant [Figure 6-3]. The ratios were logged as before, giving the following P-values; 0.65 (wild-type), 0.11 (*lig4),* 0.46 *(lig6)* and 0.28 *(lig6-1 lig4-5),* all of which indicate normally distributed values and allowing use of standard ANOVA statistical analysis.

To determine whether there was a significant difference in ratios of green and red fluorescence between wild-type *Arabidopsis* protoplasts and mutants, the students T-test was performed on the logged ratiometric data.

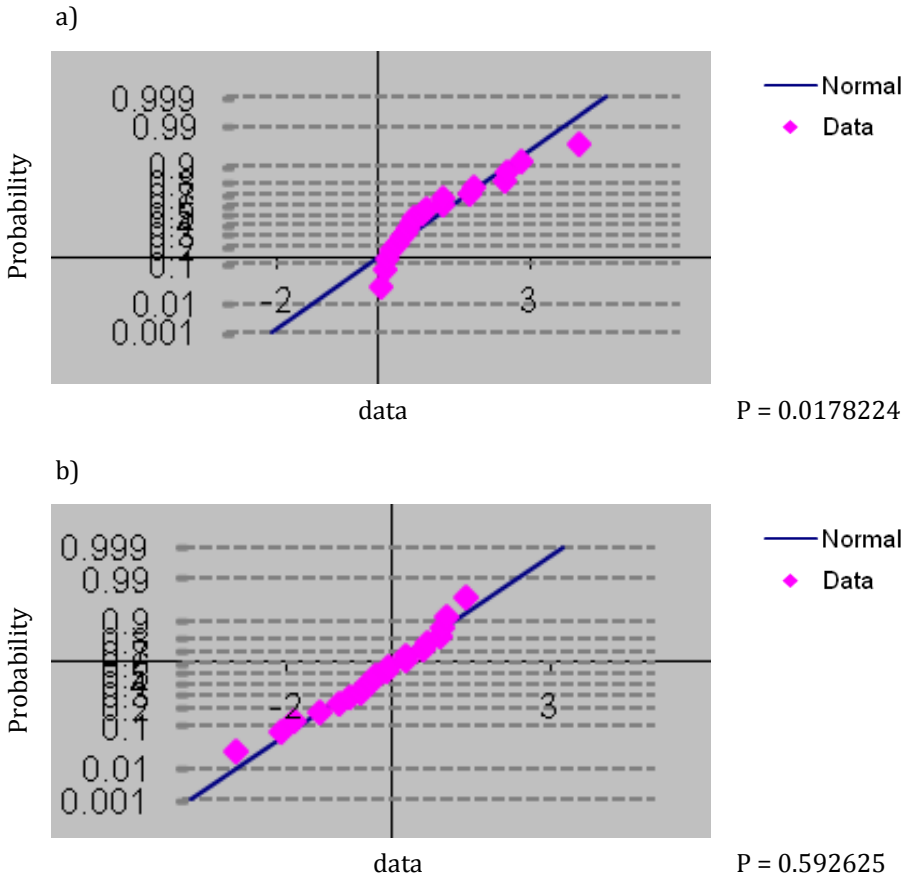


Figure 6-4 Anderson-Darling plot to determine normality of data spread

These graphs show the wild-type GFP:dsRED ratio **[A]** before and **[B]** after data has been transformed

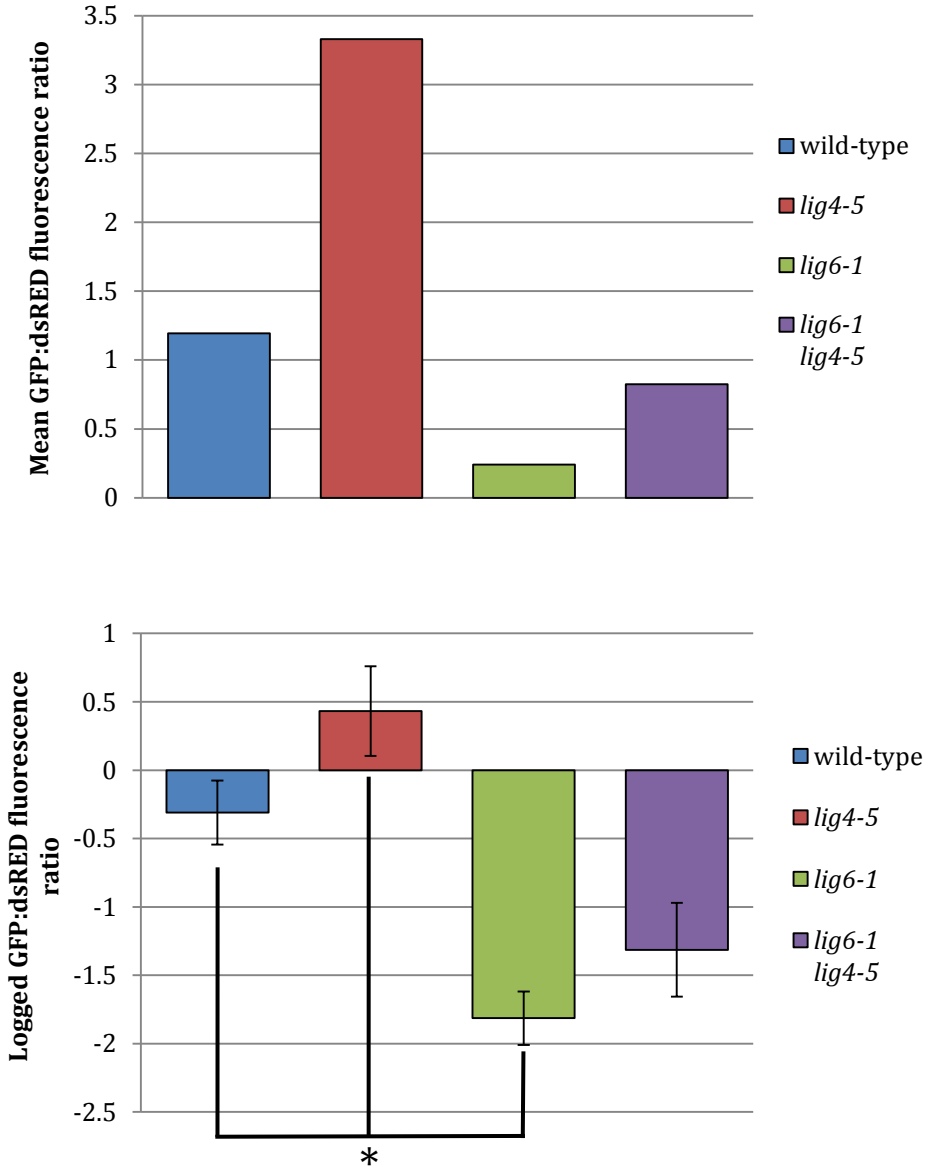


Figure 6-5 Mean average of A) GFP:dsRED ratio and B) logged GFP:dsRED ratio in *Arabidopsis* protoplasts transformed with dsRED, 5'END DEL GFP and 3'END DEL GFP.

[A] The mean ratio of GFP:dsRED is shown for wild-type; M = 1.19 (n=20), *lig4-5*; M = 0.43, *lig6-1;* M = 0.24 (n = 12) and *lig6-1 lig4-5* M = 0.83 (n = 19).

[B] The mean logged ratio of GFP:dsRED is shown for wild-type M = -0.31 (SEM =0.25), *lig4-5* M = 0.43 (SEM =0.32), *lig6-1;* M = -1.81 (SEM =0.19) and *lig6-1 lig4-5* M = -1.31 (SEM =0.34). A one-way ANOVA was performed using Tukeys HSD post hoc test to determine significant difference of the mean logged GFP:dsRED values between each genotype. Significant differences were observed between wild-type and *lig4-5* ($P = 0.043$), wild-type and *lig6-1* ($P < 0.01$) but not wild-type and *lig6-1 lig4-5* ($P = 0.13$).

The analysis showed there is a significant difference in the levels of GFP fluorescence, and therefore levels of ECR between wild-type and *lig6-1* protoplasts (P = 0.0007), with a decrease in GFP fluorescence in *lig6-1* protoplasts. A significant difference was also identified between wild-type and *lig4*-5, with increased levels of ECR in the *lig4-5* when compared to wild-type protoplasts (P = 0.04). There is no significant difference between the GFP:dsRED fluorescence ratio in wild-type and *lig6-1 lig4-5* protoplasts (P > 0.05), nor between the ratios observed in *lig6-1* and *lig6-1 lig4-5* protoplasts (P > 0.05) [Figure 6-4].

6.2.2 *LIG6* and *LIG4* protect genome stability in roots.

Plant growth is mediated by small populations of stem cells; these are located in the meristems of shoots and roots. The root meristem stem cells are called the root initials; these surround another group of stem cells collectively known as the quiescent centre (QC) (Figure 6-6). Cells in the QC divide rarely but provide an intercellular signal to the root initials preventing differentiation (Scheres, 2007). Stem cells which are displaced from centre zone of the meristem then populate the peripheral zone at which point differentiation is initiated (Stahl and Simon, 2012). Plant growth via root and shoot meristem pose particular risks, so guarding genome integrity within plant stem cell niches is vital (Heyman et al., 2013).

In the root and shoot meristems of *Arabidopsis* rapid ATM-dependent, autolytic programmed cell death (PCD) (van Doorn, 2011) ensures damaged cells are eliminated from stem cell populations (Fulcher and Sablowski, 2009; Sablowski, 2011). Confirmation that PCD is initiated as a response to DSBs in root stem cells was observed in mutants defective in the NHEJ pathway (*ku80* and *lig4*). Spontaneous PCD in roots was statistically significantly higher in NHEJ pathway deficient mutants compared to wild-type (Sablowski, 2011). Here the roles of *LIG4* and *LIG6* in maintenance of genome integrity in root meristems cells under genotoxic stress was investigated and quantified in roots exposed to bleomycin.

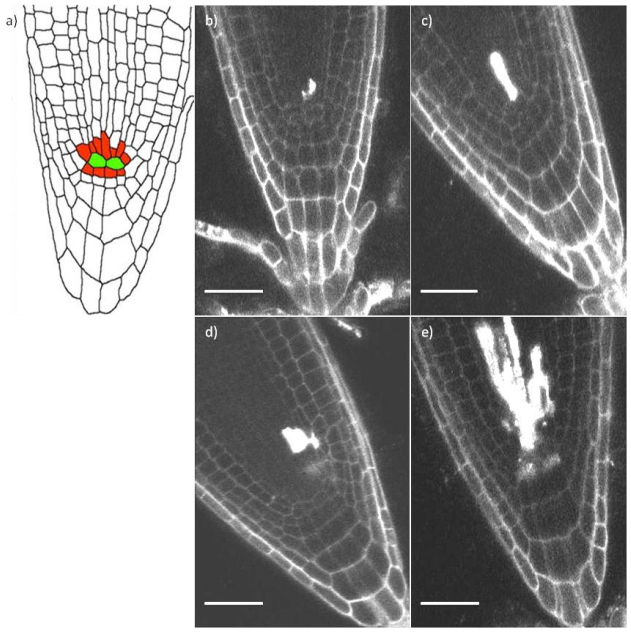


Figure 6-6 Propidium iodide (PI) staining of root tips showing PCD events in wild-type, *lig4-5, lig6-1* and *lig6-1 lig4-5* mutants after bleomycin treatment

[A] Diagram of the root meristem with the QC marked in green and the root initials marked in red, adapted from (Fulcher and Sablowski, 2009). **[B-E]** representative confocal images of 4 day old root tips including a 24 hour period of bleomycin treatment (1 µg ml^{-1}) stained with PI which enters dead cells but only marks the outline of those living, **[B]** wild-type, **[C]** *lig4-5* mutant, **[D]** *lig6-1* and **[E]** *lig6-1 lig4-5* double mutant (Scale Bar; 50 µm).

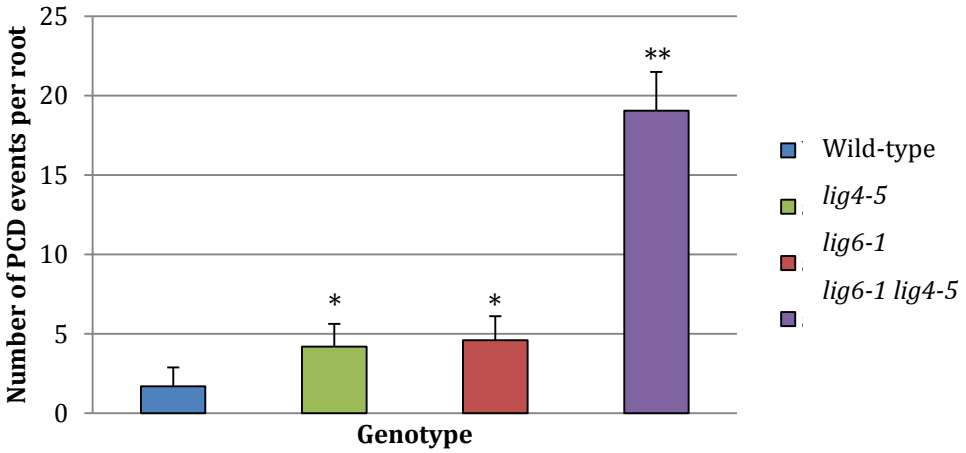


Figure 6- 7 Number of programmed cell death events per root in wild-type, *lig4, lig6* and *lig6 lig4* mutant plants

Statistical analysis using the student T-test determined a significant difference between wild-type and *lig4-5 (*P =0.00039 *); *lig6-1* (P = 0.00009 *); *lig6-1 lig4-5* (P < 0.00001 **). The standard error of the mean is displayed. (n = 20).

3 day old seedlings (wild-type, *lig4-5, lig6-1* and *lig6-1 lig4-5*) were treated with the radiomimetic drug bleomycin for 24 hours to induce DSBs. Roots where incubated in propidium iodide (PI), which stains the cell walls of living cells but is a marker for loss of cell membrane integrity and cell death when entering and staining the entirety of a dead cell (Truernit and Haseloff, 2008). Finally roots were then imaged under the microscope [Figure 6-6 B-E]. Wild-type roots were imaged (n=20) showed a mean incidence of 1.7 PCD events per root. For *lig4* and *lig6* single mutant lines the mean incidence of PCD events was similar to each other; 4.2 and 4.6 per root respectively. This is around a 2.5 fold increase in PCD incidence in both cases when compared to wild-type and are both significantly different (Students T-Test $P < 0.00001$). A mean incidence of 19 PCD events was observed in the double mutant, over an 11 fold increase from wild-type. These results show genome integrity is decreased in the *Arabidopsis* root meristem without the action of the ligases LIG4 and LIG6.

6.3 Discussion

6.3.1 The roles of *LIG4* and *LIG6* in extra-chromosomal recombination

In 1990's the first report which enabled quantification of extra-chromosomal recombination events and the rate at which they occur in plants by the use of β-glucuronidase (GUS) as a marker was published. This allowed measurement of extra-chromosomal recombination (ECR) events within a cell (Puchta and Hohn, 1991) and represents a landmark study of plant recombination mechanisms. Work with mammalian cell culture has given differing results in the roles of DNA repair factors have on rates of ECR. In human and hamster fibroblast cell culture the incidence of ECR are found to be increased in cells deficient in ATM (Drexler et al., 2004), however the number ECR events have been shown to be unaffected in mice fibroblast cell culture deficient in *KU80* (Morrison and Wagner, 1996).

The results from this study clearly showed a difference in the incidence of recombination events between wild-type, *lig4-5, lig6-1* and *lig6-1 lig4-5* mutant lines. Interestingly there was a significant increase in recombination with regards to *lig4* mutants when compared to wild-type, *lig6-1* and *lig6-1 lig4-5* lines [Figure 6-4]. An increase in GFP:dsRED ratio in *lig4-5* protoplasts when compared to wild-type suggested that ECR was down-regulated when *LIG4* was expressed. This was observed as an up-regulation of ECR activity in *lig4*-deficient protoplast*s* at an extra-chromosomal level.

A significant decrease of the GFP:dsRED ratio was observed in *lig6* protoplasts when compared to wild-type [Figure 6-5]. The GFP:dsRED ratio decreased in mutants deficient in *LIG6* and demonstrated a role for LIG6 in facilitating homologous extra-chromosomal recombination. From these results it could be hypothesised that LIG6 played a role in facilitating chromosomal recombination whilst LIG4 had an inhibitory role. Little has been reported on the role of LIG6 in plant recombination but these results demonstrated a hitherto uncharacterised role in extra-chromosomal recombination pathways.

This assay measured recombination activity on an extra-chromosomal basis. Intra-chromosomal recombination assays would be necessary to confirm a role for LIG4 and LIG6 in regulation and promotion of chromosomal recombination, but these results gave support towards such roles. Work in *rad50 Arabidopsis* mutants observed increased intra-chromosomal recombination consistent with RAD50 suppressing recombination in *Arabidopsis* (Gherbi et al., 2001)

To produce fluorescent GFP protein in this ECR assay, the two plasmids must have undergone a recombination event, requiring the recombination machinery of the plant. It is therefore interesting that a significant increase in fluorescence levels in *lig4* mutant protoplasts was observed when compared to *lig6* mutant lines. LIG4 has been well characterised and is important in the NHEJ pathway (West et al., 2000). It is possible that action of LIG4 promotes NHEJ repair before homologous recombination can occur. It is through HR creation a functional *GFP* gene would occur. Interestingly the double mutant showed levels of recombination with no significant difference to wild-type, suggesting competitive opposing action of LIG6 and LIG4. Such competition between HR and NHEJ pathways has been reported in mammalian cultures where cells deficient in the core NHEJ factor *XRCC4* displayed increased instances of HR-mediated DSBR (Delacote et al., 2002). Further to this *in vitro* studies showed competition for a common DNA substrate between the human RAD52 and KU proteins, involved in the initial steps of HR and NHEJ respectively (Van Dyck et al., 1999). A report in *Arabidopsis* showed the absence of another component of the NHEJ, RAD50, and stimulated homologous recombination in planta and is consistent with the hypothesis of competition between HR and NHEJ pathways.

LIG4 is known to be a key component of the canonical NHEJ. Work in *Arabidopsis* has defined the DNA repair kinetics of DSBs where c-NHEJ is the predominant DSB repair mechanism before back-up NHEJ pathways (section 1.3.5.2) and then homologous recombination repair (Charbonnel et al., 2010; Waterworth et al., 2009). Removal of LIG4 may decrease the efficiency of NHEJ,

allowing ECR events to occur with increased incidence, consistent with the hypothesis of competition between HR and NHEJ.

To test this hypothesis, over-expression lines of *LIG4* and *LIG6* could be created and the same assay performed with these. If competition occurs here, extra-chromosomal recombination rates will decrease in lines over-expressing *LIG4* and increase in lines over-expression *LIG6*. Furthermore analysis of mutants deficient in other core NHEJ factors, such as *KU80* may give further insight into the regulation of repair pathway choice i.e. HR or NHEJ.

6.3.2 The roles of *LIG4* and *LIG6* in genome stability in roots

Mild increases in PCD in both *lig4* and *lig6* mutants reinforced previous reports that genomic integrity is tightly controlled in the root meristem (Fulcher and Sablowski, 2009). Interestingly, an 11-fold increase in PCD events was observed in the double mutant here, significantly more PCD than that observed in the single mutants ($P < 0.00001$). This suggested there is redundancy between LIG6 and LIG4 and/or that the cumulative effects of damage resulting from the *lig4* and *lig6* mutants had additive effects on promoting PCD. Hypersensitivity to bleomycin observed in the single mutants showed that neither ligase could completely compensate for the loss of the other ligase; this result was consistent with root growth phenotypes after 100 Gy X-ray (Waterworth et al., 2010). Both ligases may be needed for efficient repair of a specific subset of DNA lesions; in *lig4* and *lig6* mutants one of the remaining ligases may function in inefficient back-up pathways. T-DNA insertion studies demonstrated that *lig6 lig4* mutants were able to perform inter-chromosomal recombination, albeit with decreased efficiency, demonstrating *LIG1* (as the only functional ligase present in this mutant line), could partially compensate for loss of both *LIG6* and *LIG4* (Park et al., 2015). This demonstrates that plants have evolved very flexible repair pathways that are able to operate without core-components.

These results are interesting with regards to understanding how a plant deals with genotoxic stress and also which cells are more susceptible to that stress. It

is known that recombination factors are important in maintaining viability in the stem cells of the meristem even when plants are grown under ideal conditions (Fulcher and Sablowski, 2009). Analysis of PCD frequencies in response to genotoxic stresses more representative of those a plant may encounter, such as high salt concentration and low moisture content, would show whether these mutants have the same phenotype under more physiologically relevant stresses. Mutant lines deficient in NHEJ factors *LIG4* and *X-RAY CROSS COMPLEMENTATION PROTEIN 4* (*XRCC4)* in conjunction with loss of another NHEJ-associated factor *DNA POLYMERASE LAMBDA* (POL λ) indicated the involvement of NHEJ-mediated repair of salinity-induced DSBs (Roy et al., 2013).

By over-expressing those recombination factors which, when deficient, showed a decrease in cell viability in *Arabidopsis* root initials, it may be possible to promote meristem cell viability and therefore increase whole plant viability (Sablowski, 2011). It was interesting to discover a redundancy in phenotypic function between LIG6 and LIG4; the question must therefore be raised: why did plants uniquely evolve *LIG6*? Further work is therefore needed to determine this and to understand the distinct roles and mechanisms LIG6 and LIG4 have in maintaining genome stability and ultimately cell viability in the root meristem. It is possible that LIG6 and LIG4 function in the same pathway as complementary factors. A second possibility is that these two proteins are phenotypically complementary but function in two separate pathways such that deficiency in both leads to a large decrease in genome stability and ultimately cell viability [Figure 6-7]. Similar analysis of other knock-out mutants of plant DNA repair factors may provide a greater understanding of the roles DSB repair plays in plant meristem maintenance.

7. General Discussion

7 General Discussion

Seed germination vigour is a major determinant of crop yield and survival of plants in the natural environment. However, our knowledge of the molecular factors important to seed quality is far from complete. Such understanding is vital to mitigating the detrimental effects of extended storage on seed vigour and viability. In particular, storage under hot and humid conditions exacerbates deterioration of seed quality with farmers in the developing world often not having access to controlled storage facilities in order alleviate these stresses to the seed. Identifying factors important to maintenance of seed vigour and longevity should enable biotechnological or plant breeding approaches to improve these qualities. In crop species, this is an obvious strategy to maintain and improve crop yields. These studies have identified important functions of both double and single strand break repair mechanisms in the maintenance of seed vigour and longevity.

7.1 Towards a well-defined model of DNA repair processes in seeds

7.1.1 NHEJ repair of DSBs is the most important DNA repair pathway in mitigating the deleterious effects of seed ageing.

Analysis of NHEJ mutants deficient in *lig4* and *lig6* identified a molecular link between seed longevity and DSB repair (Waterworth et al., 2010b). Here, further investigation of the NHEJ pathway in the context of germination was undertaken using mutants deficient in *ku70* and *ku80.* These demonstrated a lower seed vigour in high quality seeds, conforming with previous phenotypic analysis of *lig4* and *lig6* mutants (Waterworth et al., 2010). The work presented by this study provides further evidence that components of non-homologous end joining pathways have direct and important roles in seed quality. Analysis of the sensitivity of *lig4* and *lig6* to accelerated ageing identified that *lig6* played a greater role in maintenance of seed vigour and viability. The additive phenotype is suggestive that these ligases play distinct roles in germination, possibly in pathways with specificity for different end chemistries. Although KU70 and KU80 function upstream of *lig4* in the c-NHEJ pathway, the hypersensitivity of KU factors to seed ageing was more comparable to that displayed by *lig6*, potentially indicating KU might also function upstream of *lig6*. Interestingly, NHEJ-factor deficient seeds were the only mutant types investigated in this study that displayed a relative decrease in seed vigour of unaged seeds along with decreased viability after accelerated ageing comparison to aged wild-type seeds. This hypersensitivity further demonstrates a requirement for the repair of DNA DSBs in early-imbibing seeds as well as roles for the recombination machinery in the recovery from damage accumulated during quiescence. NHEJ therefore presents the greatest potential in identifying avenues to lessen the effects seed ageing. Indeed recent genetic analysis of determinants of *Arabidopsis* seed longevity identified that a QTL

confirming increased longevity co-localised with the *LIG4* locus (Nguyen et al., 2012).

To determine if NHEJ factors can promote increased seed longevity and vigour, analysis of over-expressing lines would be informative. Over-expressing *KU70* and *KU80* using seed specific promoters could increase NHEJ repair capacity during germination, potentially resulting in seeds with greater resistance to seed ageing and germination stresses.

7.1.2 XRCC2-mediated HR has a role in mitigating the effects of seed ageing but is not as important as NHEJ in seed quality

As demonstrated in chapter 4, without the homologous recombination factor *XRCC2*, germination vigour was lower in aged seeds when compared to equivalent wild-type germination performance. Unaged *xrcc2-1* seeds were shown to have germination performance indistinguishable from wild-type seed lots [Figure 4-2]. This differs from the decrease in vigour displayed by seeds deficient in the NHEJ factors *lig4, ku70* and *ku80* [Figure 3-3 A and 3-10 B]. Similarly, unlike these c-NHEJ mutants, the *xrcc2-1* lines did not show differing levels of viability to wild-type seeds after seed ageing [Figure 4-3].

In higher plants, HR mutant plants are phenotypically normal during the vegetative phase of development and do not display pronounced hypersensitivity to DSB-inducing treatments, including X-rays. It is therefore thought that NHEJ is the major pathway utilised in DSB repair in these cases (Waterworth et al., 2011). This may also explain the increased hypersensitivity in c-NHEJ mutants to ageing treatment [Figure 3-11] compared to that observed in HR mutants when compared to wild-type [Figure 4-4]. Decreased vigour observed in unaged NHEJ mutants was not present in *xrcc2-1* lines and supports this hypothesis. From this it may be deduced that HR functions to repair a specific sub-set of DNA damage lesions that are not present in unaged seeds but rather accumulated during storage. For example it is known that the HR sub-pathway single strand annealing accounts for *c.* 30% of repair events

that occur between tandem repeats (Siebert and Puchta, 2002). Indeed *XRCC2* is known to have a non-redundant role in SSA as well as RAD51-dependent homologous recombination (Serra et al., 2013). Deep sequencing of the aged seed genome in *xrcc2-1* lines (section 7.2.1), might elucidate the identity of the damage products targeted and repaired by *XRCC2*-mediated HR.

To repair a damaged region of DNA, HR requires an intact template, permitting an error-free break repair event. A homologous template is used for repair in a pathway mediated by the *RAD51* recombinase protein in conjunction with accessory proteins including the focus of part of this study, XRCC2. In yeast and mammals, HR is tightly regulated and this recombination activity is dependent on cell cycle stage. The lowest level of HR activity was observed in G1, before replication of a sister chromatid, whilst activity is highest during S-phase and G2 when sister chromatids are available (Mazon et al., 2010). Analysis of the *Arabidopsis* seed has demonstrated that the cell cycle is highly regulated during germination and was only initiated at or around the end of germination. Cells are mostly in G1 throughout imbibition when HR levels are low. DNA synthesis (S-phase) coincides with radicle emergence (Barrôco et al., 2005; Masubelele et al., 2005) and therefore during germination in *Arabidopsis,* HR may not be as important as NHEJ due the cell cycle stage of embryo cells.

In rye and *Avena fatua* (oat) seeds, DNA repair processes have been shown to start during the earliest phases of germination, only 30 minutes after imbibition initiation. This was demonstrated by ^{3}H-thymidine incorporation into DNA and unlike in the chain of germination events characterised in *Arabidopsis*, these cereals display DNA replication within several hours of imbibition prior to radicle emergence (Elder and Osborne, 1993; Barrôco et al., 2005). In these species, HR may have a more prominent role in repairing DNA lesions as the cell cycle has progressed further than observed in *Arabidopsis* during the early stages of germination. With this in mind, the relative importance of different repair pathways could vary with regards to germination across higher plant species.

Homologous recombination is a highly conserved, multistep and multifaceted pathway involving many factors (Mazon et al., 2010). In chapter 4, *XRCC2* gene was chosen as the candidate to investigate the role of HR in *Arabidopsis* seed germination due the characterised role of *XRCC2* DNA damage-mediated HR whilst not being essential for meiosis based HR. Many other HR factors are involved in *meiotic* HR and are sterile; it is therefore very difficult to study seed germination in knockout mutants such as *rad51* (Li et al., 2004). Following on from the present study, different HR factors could be examined to further probe their roles within the context of germination. Other HR factors of interest which do not display sterility in *Arabidopsis* are *RAD51B* and *RAD51D* (section 1.5.2).

7.1.3 SSB repair functions in aged seeds but was not as important as the repair DSBs to seed quality

Pre-genomic era studies identified that accumulation of SSBs correlated with seed quality (Cheah and Osborne, 1978). This study investigated the roles of two well-defined SSB repair pathways, base excision repair and nucleotide excision repair pathways, in germination. Both *ARP* and *ERCC1* have been well characterised in *Arabidopsis* and have non-redundant roles in BER and NER respectively, as demonstrated by hypersensitivity to genotoxic stresses (sections 5.1.1 and 5.1.2). Germination performances of unaged *arp* and *ercc1* mutant lines were phenotypically indistinguishable from that of wild-type seeds [Figure 5-4]. This suggested that these SSB repair pathways do not have an important function in unaged seeds. Upon accelerated ageing the MGT of *arp-1* was significantly increased from wild-type aged seeds; however *ercc1-1* mutant seeds remain indistinguishable from wild-type [Figure 5-5]. This demonstrated *ARP*-mediated BER held a more important role than *ERCC1*-mediated NER repair during seed germination. Indeed work in maize identified a four-fold increase of apurinic/apyrimidinic sites in DNA during early germination, which was attributed to an intermediate step of BER whereby DNA glycosylase activity removes damaged bases (Dandoy et al., 1987). This demonstrates the large cumulative levels of oxidative damage in the seed which

was repaired by BER early in germination. The importance of BER to seed quality is further highlighted in *Arabidopsis* where over-expression of the BER glycosylase *OGG1* results in increased tolerance to ageing treatments (Chen et al., 2012)

Whilst *arp-1* lines have lower seed vigour than wild-type seeds after ageing, the final viability was not significant different from wild-type. This phenotype was the same as that observed in the *xrcc2-1* mutant lines (section 7.1.2) but differs from the NHEJ mutants where ageing treatment causes significant decrease in viability (section 7.1.1). This suggested that NHEJ pathways have a more significant role in maintaining seed quality than those involved in SSB repair. This was most likely due to the nature of the lesions repaired by each pathway. DSBs are highly cytotoxic DNA lesions due to their highly mutagenic nature. Chromosomal fragmentation results in the loss of large chunks of genetic information and this explains why plants cannot tolerate high levels of DSBs (section 1.5) (Waterworth et al., 2011). Oxidative damage to DNA bases may have serious consequences for the cell but is generally better tolerated (section 1.3.3) (Roldán-Arjona and Ariza, 2009), however, the mutation rate due to seed ageing is unknown (Section 7.2.1).

7.1.4 The potential roles of other DNA repair mechanisms in seed quality

Direct reversal of UV induced photoproducts by photolyase enzymes is an important DNA repair mechanism in green tissues (Waterworth et al 2002). Further work following on from the present study could seek to study the photoreactivation pathway to elucidate any roles in seed quality. It is probable that this pathway only has a minor role in the repair of the genome in germinating seeds under normal physiological conditions. This is due to action of photolyase enzymes being light-dependent (Dany et al., 2001). As seeds are under soil whilst imbibing and proceeding through germination they are exposed to limited amounts of light. Seeds may accumulate UV-induced photoproducts during maturation on the mother plant. However, NER mediates slower ‘dark repair’, of photoproducts (Fidantsef et al., 2000), which could

repair such damage upon imbibition. Analysis of *ercc1* and other NER factor mutants in germination performance in the dark would determine if NER repairs photoproducts in seed germination.

A further avenue of investigation to identify the roles of mismatch repair (MMR) in germination would allow determination of potential roles in seed germination. Work in *Arabidopsis* has shown that unaged seeds deficient in *MSH2* show germination indistinguishable from wild-type in the first generation. These mutants do however display increased endogenous microsatellite instability and fifth generation seed lots displayed variable germination vigour and seedling establishment compared to wild-type (Hoffman et al., 2004). This suggests that in unaged *Arabidopsis* seeds MMR does not play a role in germination, but has essential roles in maintaining genomic integrity to ensure deleterious mutations are not accumulated and passed on to the progeny.

MMR is known to primarily be involved in base-base mismatches and insertion/deletion mis-pairs generated during DNA replication and recombination (Li, 2008). In *Arabidopsis* seeds, flow cytometry has revealed that a major transition through S phase toward G2 is only detected around the moment of radicle emergence and therefore completion of germination (Barrôco et al., 2005). It should be noted upon 8 hours of imbibition in water, flow cytometry data shows a peak corresponding to 4C nuclei in *Arabidopsis* seeds but only after 40 hours after imbibition is a large increase in the frequency of 4C nuclei observed coinciding with radicle production (Barrôco et al., 2005). This suggests that the majority of DNA synthesis involved in replication occurs at or around the completion of germination, coinciding with radicle emergence. It can hypothesised that in *Arabidopsis*, the mismatch repair pathway does not play an important role in DNA repair in seeds during phase I and II of germination *sensu stricto* as little replicative DNA synthesis is present until the end of germination.

7.1.5 Roles of alternative repair pathways in germination

Several pathways which mediate DNA repair are conserved across eukaryotes and have been well defined in plants, such as the c-NHEJ pathway. However, it is becoming clear that plants, as other organisms, have multiple back-up repair pathways that facilitate repair using different components of the DNA repair machinery (Charbonnel et al., 2010).

Plants have a relatively high tolerance for DNA damage and mutations in characterised repair pathways are viable. This is suggestive of alternative repair pathways having higher activity in plants than animals (Waterworth et al., 2011). An increased understanding of the conservation and evolution of DNA repair pathways between plant species may provide further insights into the relationship between DNA repair and seed quality. Work using comparative genomic analysis between *Arabidopsis* and rice as well as by performing similarity searches and conserved domain analysis against proteins known to be involved in DNA damage repair in human, yeast and *E. coli* has been previously reported. It was found that core components of all repair pathways are generally conserved across eukaryotes but there are some divergences. NER and MMR were found to be the most highly conserved pathway (Singh et al., 2010). Interestingly, plants contain homologues of both the animal *OGG1* and bacterial *FPG* glycosylases, which could reflect exposure to the particularly high levels of oxidative stresses experienced by plants due to their sedentary lifestyle. Several novel genes, for instance *MSH7* and *LIG6*, not present in other eukaryotes, are found to be well conserved across higher plants (Singh et al., 2010; Waterworth et al., 2010). This supports the hypothesis that plants have evolved novel mechanisms to maintain genome stability.

The desiccation tolerant seed represents the evolution of a very efficient mechanism of reproduction. The embryo genome is potentially exposed to high levels of genotoxic stress during seed development and maturation on the mother plant and in soils with unfavourable conditions such as high salt or acidity levels. Other desiccation tolerant organisms have also evolved novel approaches to mitigate genome damage. The bacterium *Deinococcus*

radiodurans is known for its resistance to extremely high doses of ionizing radiation and for its ability to reconstruct a functional genome from hundreds of radiation-induced chromosomal fragments. It is thought this capability evolved to ensure survival during desiccation which is associated in this bacteria with an accumulation of DSBs (Mattimore and Battista, 1996). It would be expected that plants have evolved repair activities to cope with DNA damage in the seed stage of the plant lifecycle , as exemplified by the isolation of novel repair factors such as *LIG6* (Waterworth et al., 2010). This highlights the importance of understanding the physiological relevance of different DNA repair pathways and their importance at different stages of the plants lifecycle.

7.1.6 Functions of DNA Ligase 6

Most of our knowledge of plant DNA repair mechanisms arises from studies in which plants are exposed to non-physiological stresses (Waterworth et al., 2011). An important aspect of the work presented in this study was the identification of roles for several DNA repair pathways in seed biology, expanding our understanding of the physiological functions of plant DNA repair mechanisms. For example, *LIG6* was first phenotypically characterised due to its role in seed germination (Waterworth et al., 2010), unlike *LIG4,* which is well characterised as part of the c-NHEJ pathway in *Arabidopsis* (West et al., 2000). The plant specific *LIG6* has a highly conserved ligase domain and N-terminal domain with homology to the SNM/Artemis class of proteins. This could suggest a role in repair or end processing of DSBs with a particular end chemistry, such as ARTEMIS in mammalian c-NHEJ (Lieber, 2010) This could be tested by over-expression of recombinant *LIG6* in *E. coli* and via biochemical *in vitro* assays of activity with various DNA substrates.

Whilst the pathway in which *LIG6* operates has yet to be delineated, work in this study demonstrated a role for *LIG6* in extra-chromosomal recombination, functioning in direct competition with *LIG4.* Better understanding of the *LIG6* pathway in plant DSB DNA repair could specifically elucidate the specific types of DNA lesions that most affect seed quality. *LIG6* was first identified in plants

due to sequence homology to *LIG1* but it also contained the catalytic core of eukaryotic PSO2/SNM1/ARTEMIS proteins (section 6.1.2). It maybe that *LIG6* is functional homologue of these proteins (section 1.5.3.1) whereby LIG6 binds to a hairpin structure and either nicks/facilitates nicking of the hairpin. mutant lines deficient in *LIG6* line displays redundancy of this function suggesting it is not the primary function *LIG6* (Huefner et al., 2011).

Delineating the pathway in which *LIG6* functions may be possible through identification of interacting proteins using LIG6 as bait in yeast two-hybrid system library screening. Alternately tandem affinity purification (TAP) tagging and mass spectrometry (MS) analysis of proteins interacting with epitope tagged LIG6 could be employed. These approaches have been previously used to successfully identify novel interactors of core plant NHEJ repair pathway components. For example yeast two-hybrid system library screening demonstrated the interaction of MRE11 and the histone acetylase TAF-1 in *Arabidopsis* (Waterworth et al., 2015). This approach may elucidate interacting proteins of LIG6 as well as revealing further novel DNA repair.

7.1.7 DNA repair and seed vigour

In addition to repair mechanisms, DNA damage signalling and response factors play a crucial function in the cells response to genotoxic stress. The DNA damage response in eukaryotes is controlled and coordinated by the checkpoint kinases ATAXIA TELANGIECTASIA MUTATED (ATM) and ATAXIA TELANGIECTASIA AND RAD3 RELATED (ATR). These kinases activate repair factors, halt or slow progression of the cell cycle and control pathways to cell death. Control of the cell cycle is imperative in ensuring that damage to the genome is repaired before DNA replication in order to prevent transmission of potentially mutagenic damage to the daughter cell. Indeed cell cycle progression is tightly monitored by cell cycle checkpoints and slowed or halted in the presence of DNA damage (section 1.6.3).

Decreasing vigour is accompanied by progressive delays in radicle emergence and eventually culminates in loss of viability. The molecular basis for the delay to germination observed in DNA mutants with diminished repair capacity in

these studies has yet to be elucidated. Although *Arabidopsis* seeds cells do not undergo mitosis until the point of radicle emergence, cell cycle checkpoints have been shown to be important for seed vigour. Loss-of-function alleles of 'early-activated' CYCDs during phase I of germination, display reduced division activation and consequential delayed root emergence (Masubelele et al., 2005). This demonstrates a potential mechanism by which seeds are able to delay germination whilst repair is on-going. ATM and ATR are the two master kinases which co-ordinate the DNA damage response and are responsible for inhibiting cell cycle progression in response to DNA damage [Figure 1-9 (Culligan et al., 2006; Matsuoka et al., 2007; Roitinger et al., 2015)]. To test this hypothesis the roles of ATM and ATR in seed germination should be analysed. The MGT is increased wild-type *Arabidopsis* seeds that have undergone accelerated ageing, demonstrating lower seed vigour. If either ATM or ATR function to halt cell cycle progression in response to DNA damage in seeds, this may potentially this would cause subsequent delay in the radicle emergence. Mutants deficient in these genes would potentially display unaged vigour phenotypes even after ageing. If such a phenotype was observed, this work would further elucidate the importance of the DNA damage response and repair processes in seed quality. If removal of this regulation causes a decrease in MGT, then this would demonstrate regulation is not mediated by other forms of damage such as that to proteins or lipids which is causing lower seed vigour (section 7.3).

7.2 DNA damage accumulation in seeds and the molecular consequences

A decrease in seed quality can be attributable to effects of ageing on the repair capacity. Impairment of enzyme functions due to protein damage exacerbates accumulation of DNA damage products exceeding repair capacity. In addition to determining the roles DNA repair during seed germination and their roles in seed quality. Understanding the forms of genomic damage accumulated in seed ageing would help to further determine the roles of each repair pathway in the germinating seed in the context of impaired repair capacity.

7.2.1 Accumulation of DNA damage during seed storage

The main focus of these studies has been the roles played by representative factors for each DNA repair pathway under normal germination conditions with or without accelerated ageing. It remains unknown whether these conditions cause a wide spectrum or specific subsets of DNA damage lesions. Quantitative analysis of DNA damage products present in the embryo both before and during imbibition would indicate what type of damage products are accumulated by the dry seed. Similarly, the factors analysed here may have important roles in germination when the seeds are exposed to different genotoxic stresses.

In order to investigate the types of DNA damage in the dry seed, further work could be conducted utilising the single cell gel electrophoresis (comet) assay. This has been adapted to differentiate between SSB and DSB repair in genomic DNA and successfully applied to a number of plant species including Arabidopsis (Kozak et al., 2009). Applying this technique to aged and unaged embryonic seeds cells could elucidate the relative amount of DSBs and SBB accumulated after ageing. However DSBs are extremely cytotoxic and the relative number observed even after some seed ageing that lowers vigour maybe low and not quantifiable using the comet assay. Chromatin remodelling is known to be important in the cellular response to DNA DSBs. Rapid phosphorylation of the histone H2AX to γ-H2AX, a widely used biomarker for

DSBs, may provide a quantitative measure of DSB events in the seed embryo. Visualising the appearance and loss of γ-H2AX foci by using immunostaining with anti-γ-H2AX has been shown to be a reliable quantitative measure of DSB events and repair kinetics in *Arabidopsis* (Charbonnel et al., 2011).

A major type of oxidative damage observed in the genome is 8-oxoG (section 1.3.3). It is possible to quantify the levels of 8-oxoG in the genome of the embryo by either an enzyme-linked immunosorbent assay (ELISA) or High-Performance Liquid Chromatography (HPLC) using Anti-8-oxo-dG (Yoshida et al., 2002). This would identify levels of oxidative base damage in the embryo and could determine if there are threshold levels of oxidative damage at which seed quality deteriorates.

The accumulation of mutations upon ageing may represent a significant factor in seed quality. Genome stability could be evaluated by performing genome sequencing of unaged and aged wild-type seeds and comparing wild-type to the mutant lines described in these studies. This work would identify not only the levels of mutations caused by seed ageing, but also which repair pathways are important in maintaining genome integrity in the seed. Furthermore, mutations in the genome may preferentially occur at specific loci. Work in mammalian cancer cells has identified so called 'common fragile sites' (CFS). These genomic loci are evolutionarily conserved late replicating regions with AT-rich sequences, which have increased genomic instability associated with cancer (Ma et al., 2012). Using sequencing analysis would potentially elucidate hotspots within the *Arabidopsis* genome with increased genome instability after seed ageing.

7.2.2 The importance of telomere maintenance in seed quality

Repair and maintenance of telomeres during germination may also represent a role for DSB repair mechanisms in germination. In eukaryotes, chromosomal ends are linear and are protected from recognition as broken DNA ends by highly conserved nucleoprotein telomeric cap structures. The DNA damage response can be triggered if telomeres are unprotected and can form substrates

of recombination pathways. This can result in mutagenic chromosomal rearrangements and fusions, ultimately leading to cell death (Amiard et al., 2014). Between species, varying lengths of TTTAGG tandem repeats usually form plant telomeres. Shortening lengths of telomere below *c.* 250 nucleotides is associated with genomic instability in *Arabidopsis* (Heacock et al., 2004). After seed ageing, the loss of viability in wheat grain is preceded by the fragmentation of wheat telomeric sequences (Bucholc and Buchowicz, 1992, 1995). Accumulation of genomic lesions or nucleolytic degradation during the quiescent state of the dry seed could cause telomere loss and lead to deletional recombination events (Watson and Riha, 2011). *Arabidopsis ku70* mutants display deprotection of telomeres with dramatic deregulation of telomere length control. Mutant telomeres were observed expanding to more than twice the size of wild type by the second generation (Riha et al., 2002). The hypersensitivity of these mutants, specifically at the seed stage of the plant lifecycle may provide a molecular explanation as to why germination vigour loss is observed in the NHEJ mutants. The activity of this complex correlates with DNA replication and is absent in dry *Melandrium album* seeds (Riha et al., 1998). In contrast, aged wheat seeds display telomere restoration within 90 minutes of imbibition (Bucholc and Buchowicz, 1992). Further insight into the regulation and loss of telomere integrity may provide an avenue into understanding causes of DSBs and associated chromosomal defects during seed ageing.

7.2.3 Protection of DNA against damage in seeds

The studies presented here have focussed on the repair of accumulated damage to the genome once metabolism in the seed is re-established after imbibition. Understanding the protective mechanisms which minimise accumulation of DNA damage in seeds also provides an important aspect of our understanding in seed quality and the molecular basis for DNA damage and repair.

The role of the seed coat is important to seed quality. Germination performance is reduced after natural and accelerated ageing in mutants that exhibit testa defects (Debeaujon et al., 2000; Clerkx et al., 2004). Aged rice seeds have

increased testa permeability due to physical damage. These seeds have increased sensitivity to aluminium genotoxicity compared to unaged seeds (Alvim et al., 2007). This demonstrates the role the testa holds in protecting seed embryos from exogenous stresses. Understanding the genetic and molecular events that take place during testa development may allow breeding of seeds with testa that harbour more effective characteristics required for seed longevity through added protection of the seed genome.

Despite the quiescent nature of dry seeds, plants possess effective mechanisms to minimise excessive oxidative stresses and curtail the accumulation of genomic damage. In orthodox seeds a number of proteins are associated with desiccation tolerance and maintenance of the quiescent state. The abundance of seed storage proteins, late embryogenesis abundant (LEA) proteins and heat shock proteins (HSPs) strongly correlate to seed longevity (Rajjou and Debeaujon, 2008). HSPs have an important role in protein stability and folding acting as a molecular chaperone. HSPs also act to protect other proteins from oxidative damage. In transgenic *Arabidopsis* lines that over-express heat stress transcription factors, subsequent increased accumulation of HSPs an augmented tolerance to seed ageing is observed (Prieto-Dapena et al., 2006).

During imbibition, detoxification systems in the seed include a number of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), (Bailly et al., 1996; Bailly, 2004; Bailly et al., 2004; Rajjou and Debeaujon, 2008), which act to mitigate the effects of oxidative stress to cellular macromolecules and organelles. ROS is known to attack DNA, causing damage to the genome and potentially introducing mutagenic changes (section 1.3.3). Understanding how these systems protect the genome is essential in understanding how the seed maintains quality and viability.

7.3 The relative importance of DNA damage in seed deterioration

Seed quality is defined in terms of viability, which is the maximum germination capacity of a seed lot. Seed vigour, describes the speed and uniformity of germination and seedling vigour. A decrease in seed quality is intrinsically linked to lower vigour, viability and a greater susceptibility to exogenous stresses (section 1.1.4). The work presented in this study has focussed on the role of genome deterioration in seed quality and the importance of numerous repair factors in maintenance of seed longevity and vigour. DNA repair mechanisms are major determinants of seed vigour and longevity. In particular repair of DSBs by NHEJ is the most important pathway for maintenance of seed vigour and viability, exemplified by the NHEJ-factors, *KU70* and *KU80* (section 3.2.2). However, DNA damage cannot be considered in isolation. Membranes and biological macromolecules also undergo progressive deterioration in the dry quiescent state. Protein oxidation can potentially reduce the repair capacity of enzymes, whilst membrane damage can promote degradation of nucleic acids through release of nuclease enzymes.

7.3.1 Accumulative damage to proteins and repair upon imbibition influences seed quality

Studies that have begun to elucidate molecular determinants of seed quality have demonstrated that accumulation of DNA damage and its repair alone is not the only factor. Severe deterioration of proteins in the seed results from both reduced cellular maintenance in the quiescent state and cycles of desiccation and rehydration (Oge et al., 2008). Increased levels of reactive oxygen species are observed during germination and protein oxidation occurs during imbibition (Bailly, 2004; Job et al., 2005; Kranner et al., 2010b). In *Arabidopsis* seeds, the predominant targets of oxidation are carbonylation of the highly abundant seed storage proteins and core metabolic enzymes (Job et al., 2005; Arc et al., 2011). These storage proteins may function to protect metabolic enzymes by 'mopping up' oxidative ROS molecules associated with

germination (Arc et al., 2011). Mechanisms involved in protein repair correlate with seed longevity in *Arabidopsis* including methionine sulfoxide reductases (MSR) that repair oxidative damage to methionine residues (section 1.1.4). Levels of MSR also correlate with seed longevity in varieties of *Medicago truncatula* (Châtelain et al., 2013), demonstrating this repair mechanism is important across species. Repair of abnormal L-isoaspartyl residues accumulated after seed ageing to the physiologically normal L-aspartyl is also important. PIMT1 is a protein that catalyses this conversion and over-expression confers higher seed vigour and increased longevity (Dinkins et al., 2008) (section 1.1.4).

7.3.2 Deterioration of storage RNA in the dry seed may influence seed vigour

As well as mechanisms that repair protein damage, germination performance is linked to RNA damage. Turnover of mRNA influences germination progression (Holdsworth et al., 2008). As a consequence of its single-stranded nature and cytoplasmic location, RNA is particularly susceptible to oxidation by ROS. Damage to RNA can cause a block in translation, which acts as a signal within the cell to target the mRNA for degradation (Bazin et al., 2011). In after-ripened sunflower seeds oxidation of specific mRNAs was identified, mainly encoding genes with putative functions in cell signalling including factors that may be involved in dormancy regulation. Targeted oxidation resulted in 8-oxoG damage in 24 specific transcripts (Bazin et al., 2011). This means in the quiescent seed that the levels of mRNA transcripts can be controlled non-enzymatically utilising this targeted oxidative damage (El-Maarouf-Bouteau et al., 2013).

7.3.3 Decreases in organelle integrity is associated with loss of seed vigour and viability

Seed deterioration is also associated with membrane damage and disruption of organelles. Cytological studies of aged seeds identified that disruption of mitochondria correlated with loss in seed vigour and subsequent observations

found a reduction in nucleotide levels in wheat embryos (Berjak and Villiers, 1972; Standard et al., 1983). Accumulation of lipid damage correlates with seed quality. Loss of membrane integrity associated with lipid peroxidation is accompanied with a decline in seed vigour and viability (Bewley et al., 2013). Molecular mechanisms such as antioxidants guard against the deleterious effects that ROS has on organelles and membranes within seeds. *Arabidopsis* mutants deficient in the lipophilic antioxidant vitamin E (tocopherol) display hypersensitivity to seed ageing (Sattler et al., 2004). Further to this, QTL analysis in *Arabidopsis* for seed longevity found a QTL for this trait co-localised with the vitamin E locus (Nguyen et al., 2012) and elevated levels of tocopherol in rice seeds conferred resistance to accelerated ageing and gave rise to high vigour seedlings (Hwang et al., 2014).

7.3.4 The importance of repairing DNA damage in seeds compared to other types of cellular damage

Understanding fully the molecular basis for seed quality is vital for the production of crop species with enhanced germination vigour and resistance to seed ageing. The work reported in the study has identified multiple molecular components which promote these qualities, but it must be considered in the wider context of factors which influence seed quality. It remains unclear which aspects of molecular damage are of greatest importance in the production of higher vigour seeds resistant to seed ageing. However, increasing evidence is demonstrating that effective DNA repair is essential in the production and maintenance of high seed quality.

Damage to DNA differs from damage to other components of the cell. If proteins or lipids are unrepairable, replacements can be synthesised: however replacement of damaged DNA is not necessarily as straightforward. The most cytotoxic form of DNA damage are DSBs and NHEJ significantly facilitates the repair of such lesions in higher plants (Waterworth et al., 2011). NHEJ is intrinsically mutagenic, meaning that even if a DSB is repaired the DNA sequence is potentially changed with possible detrimental effects. A blueprint for life is contained within the plant genome and mutations to this template will

be permanent. Using the genome, factors involved in repair can be synthesised and replacements for damaged molecules produced. However, without a stable genome this is not possible, emphasising the importance of genomic stability and accurate repair in seed vigour and longevity.

7.3.5 DNA damage and programmed cell death in the seed

DNA damage is toxic to the cell. In the presence of unrepairable damage, pathways to cell death can be activated as an integral aspect of the plant DNA damage response. Work in *Arabidopsis* roots demonstrated that a marked increase in stem cell death mediated by ATM and ATR was observed in mutants deficient in the NHEJ factors *lig4* and *ku80* (Fulcher and Sablowski, 2009). This further demonstrates the potential importance of DNA repair in maintaining seed viability. PCD events induced in response to unrepairable levels of DNA damage could represent the molecular mechanisms behind the observed decrease of viability in NHEJ mutants reported in this study (section 3.2.2.5) and previously in *lig4* and *lig6* mutants (Waterworth et al., 2010). To test this hypothesis, mutant seeds that have been aged and display decreased viability in comparison to wild-type could be analysed for PCD events. Removal of the embryo from the seed coat, treatment with propidium iodide and analysis utilising microscopy would determine the number of PCD events in each genotype. If increased levels of PCD events are observed in the mutant embryo compared to wild-type, this would demonstrate a potential molecular mechanism by which these seeds are more susceptible to viability loss after ageing. This would agree with the exhaustion hypothesis whereby seeds have a finite repair capacity and it is thought, when protection and repair mechanisms eventually fail, that cell death and ultimately, seed death are the result (Kranner et al., 2010a). Work in this study demonstrated the importance of the DSB repair factors *LIG4* and *LIG6* in the root meristem of *Arabidopsis* consistent previous studies in *LIG4* and *KU80* (Fulcher and Sablowski, 2009). This demonstrates the stringent regulation of DSB repair in plant stem cells and the subsequent role of PCD in maintaining the plant germline. To date, the molecular factors which regulate loss of seed viability remain unknown, but

genomic damage to embryonic meristems may be an important factor in seeds. Thus PCD may ensure the genomic integrity of the germline through selective elimination of cells with defective genomes, but excessive amounts of cell death may eventually inhibit the seeds ability to germinate at all.

7.3.5 Understanding the relationship between seed dormancy and DNA repair

Dormancy is an adaptive mechanism that seeds have evolved to prevent germination under unfavourable environmental conditions for seedling establishment. Regulation of dormancy in different species and ecotypes within species has adapted in various habitats to respond to different environmentally specific cues such as temperature and light (Finch-Savage and Leubner-Metzger, 2006; Graeber et al., 2012; Rajjou et al., 2012).

The *Arabidopsis* ecotype, Col-0, used in this study was non-dormant if grown under normal conditions and left to after-ripen for two months. The potential presence of dormancy in the aged seed lots could influence interpretation of these results. In the NHEJ mutants, a decrease in final germination potential is observed after accelerated ageing [Figure 3-11]. To ensure this was due to loss of viability and not a form of induced dormancy, a dormancy breaking treatment was given to these aged seeds lots. The results demonstrated that the decrease in germination potential was not linked to dormancy [Figure 3-14].

Whilst no dormancy was observed in this study, links between seed dormancy and DNA repair have long been established. Remaining in a hydrated, rather than desiccated state, may provide some advantage to a seed under certain environmental conditions. Metabolic activity is vastly higher in dormant hydrated seeds compared to those in the dry state (Rajjou et al., 2012). Therefore such seeds have a greater potential for active repair of DNA damage. Indeed it has been shown that hydrated, dormant lettuce seeds accumulated little chromosomal damage and retained germination vigour for extended periods in comparison to dry stored seeds (Villiers, 1974). DNA repair synthesis is also observed in dormant hydrated oat embryos (Elder and Osborne, 1993). It is probable that genome repair mechanisms are activated in

dormant seeds during wet-dry cycling in the soil during unfavourable conditions. More recently, the *Arabidopsis* ecotype Cvi, (which acquire primary dormancy during development) was found to have the highest level of *LIG6* transcripts in dormant seeds after prolonged periods of hydration (Waterworth et al., 2010). However, the molecular mechanisms of DNA repair activity in the hydrated, dormant seed remain to be established.

Interestingly QTL analysis in *Arabidopsis* uncovered a negative correlation between dormancy and seed longevity. It was demonstrated that seeds which deteriorate more rapidly in dry storage exhibiting a higher dormancy capacity than those, which display better longevity (Nguyen et al., 2012). This discovery suggests that seed longevity and dormancy could represent distinct adaptive mechanisms that have been evolved in response to varying climates to prolong embryo viability.

7.4 Implications for understanding DNA repair in germinating seeds

A large body of work in the pre-genomic era correlated seed vigour with chromosomal aberrations and SSBs (section 7.2). Understanding the molecular basis of DNA repair in seeds will also identify novel approaches for assessment of seed quality and commercial pre-germination treatments for its improvement.

7.4.1 Activation of DNA damage repair mechanisms and understanding the molecular basis for priming

Seed priming is a widely used commercial pre-sowing treatment to improve seed vigour in the field. These treatments can increase seedling field emergence in many agricultural species by as much as 5 – 10 %. It is thought that these treatments hydrate and re-establish cellular repair metabolism whilst inhibiting completion of germination (Burgass and Powell, 1984). However, despite the wide commercial use of such treatments, relatively little is known about the molecular basis of priming.

This study investigated the effects of osmopriming on mutants deficient in *lig6* and *lig4*. These DNA damage repair mutants have lower vigour than wild-type in aged seeds and display hypersensitivity to accelerated ageing (Waterworth et al., 2010). After priming treatments of unaged seeds neither mutant displayed a significantly different MGT to wild-type Figure 3-5 B], demonstrating seed vigour in these mutants can be recovered to wild-type levels by priming. Two competing hypotheses could explain the observed improved seed vigour. The first is that priming treatments allow resumption of DNA repair metabolism and that back-up DSB repair pathways are operative in DSB repair deficient mutants (section 1.5.3.2), repairing DNA damage that is associated with lower seed vigour (section 1.7). The second is known as the advancement of germination hypothesis, whereby priming treatments promote germination processes and possibly by-pass normal events associated with germination, in this case repair of DNA damage.

DNA repair synthesis during priming has been observed across many different plant species (Burgass and Powell, 1984; Ashraf and Bray, 1993; Thornton et al., 1993). A priming-like repair activity was identified in nature, where partial hydration of seeds from the desert species *Artemisia* during the night by dew was associated with a large decrease in DNA fragmentation (Huang et al., 2008). These reports support the hypothesis that priming facilitates metabolic activation of DNA damage repair responses, mitigating the loss of repair capacity in the *lig6* and *lig4* mutants. After accelerated ageing priming confers a wild-type germination vigour in the *lig4* mutants, conversely *lig6* mutants still have lower vigour than wild-type [Figure 3-5 D]. This result confirms that *LIG6* is crucial for maintenance of germination vigour and suggests that back-up repair pathways are inefficient at repairing the types of DNA damage usually facilitated by LIG6.

Whilst priming treatments used in this study reduce the MGT in all mutants and wild-type, after accelerated ageing and subsequent osmopriming viability is significantly reduced in the mutant lines. This indicates a disadvantage of using such treatments with very low quality seeds that have a reduced repair capacity. Previous work in lettuce showed that, whilst that priming treatments do advance germination, once seeds are re-dried increased susceptibility to deterioration in storage is observed (Tarquis and Bradford, 1992). Identification of other repair processes that are activated and function during priming treatment will give further insights into the molecular basis for priming and will enable better targeted priming treatments of different seed lots.

7.4.2 Biomarkers of seed quality

To better understand the specific role DNA repair pathways have in seed germination, further investigation into the molecular mechanisms which regulate these pathways should be undertaken. The DNA damage response in plants is coordinated by the ATM and ATR kinases. ATM specifically controls a dynamic and rapid transcriptional response to DSBs which facilitates repair processes (Culligan et al., 2006). *Arabidopsis* seeds early in imbibition are not undergoing extensive DNA replication. The DNA damage response transcriptional response is therefore evidently activated in imbibed seeds in response to genomic damage accumulated during storage, which is consistent with previous observations of increased frequencies of chromosomal breaks in aged seeds (Abdalla and Roberts, 1969; Roberts, 1972; Dourado and Roberts, 1984).

The latest official figures show that the value of overall output of crops is £9.4 billion in the UK alone (https://www.gov.uk/government/news/farming-industry-income-rises). The seed industry is reliant on germination testing to validate the quality of seed lots supplied to farmers. It is imperative that farmers can trust the quality of the seeds they sow to ensure good crop yields. The ability to determine the quality of a seed lot means farmers can have confidence in the seeds they sow. Germination performance strongly correlates with eventual crop yield and used in many crop species as a reliable marker of seed quality (Matthews et al., 2012). However performing this analysis can be labour intensive and time consuming depending on the MGT of the plant species. Identifying biomarkers that correlate to seed quality may provide a more efficient measure than manual germination testing.

From our understanding of the mechanisms of DSB repair, the DSB transcriptional response represents a potential candidate a molecular marker for seed quality. This study has collated and built on previous reports that DNA repair capacity and accumulated DNA damage are important determinants of seed quality. At present there is a lack of universal biomarkers for seed quality. As the DSB transcriptional response is highly conserved the same analysis may be performed across species (Waterworth et al., 2015). Work in *Arabidopsis* has shown the DSB transcriptional response occurs rapidly after imbibition peaking at only 3 hours (Waterworth et al., 2010). The more damage accumulated by the seed, the greater the DSB transcriptional response, meaning analysis is quantifiable (Waterworth et al., 2011). Transcriptional data is amenable to high-throughput analysis and is also relatively rapid and cheap to perform (Kim, 2006; Waterworth et al., 2011). These qualities make the DSB transcriptional response a prime candidate for further investigation in developing biomarkers for seed quality.

7.5 Conclusions

Seed germination vigour is a major determinant of crop yields (Matthews et al., 2012; Rajjou et al., 2012). Elucidating the molecular mechanisms that control germination vigour could therefore represent a significant step to improving crop yields in the context of climate change and hostile environments. The work presented in this thesis builds upon pre-genomic era studies that indicated a correlation between the amount of DNA damage accumulated in the embryo genome and seed ageing. The importance of the major plant DNA repair pathways to germination and seed quality was investigated though analysis of *Arabidopsis* mutants. These studies established important roles for both DSB and SSB repair factors in seed longevity and vigour. Under the conditions utilised in these investigations, only one DNA repair factor (ERCC1) out of seven analysed, did not have a role in seed vigour after seed ageing. This indicates that multiple DNA repair pathways are integral components of germination processes and important determinants of seed quality. These studies concluded that repair of DSBs by NHEJ (*KU70* and *KU80*) is the most important determinant of seed quality with regards to DNA repair.

The importance of NHEJ over other repair pathways to seed vigour is mostly likely due to the severe cytotoxicity of DSBs when compared to SSBs and the predominance of NHEJ over HR repair in higher plants. As such, work in these studies has demonstrated that further analysis of the NHEJ pathway in germination could reveal prime candidates for potential crop improvement by employment of biotechnological methods. Moreover, these studies establish important physiological roles for plant DNA repair pathways. Further studies characterised the novel plant specific *LIG6,* which has important roles in seed longevity. This work demonstrated that LIG6 functions to facilitate extra-chromosomal recombination in a competitive manner with LIG4 and is important to root meristem genome stability and viability.

The DNA damage transcriptional response in *Arabidopsis* to DSBs is exhibited very early in phase I of germination and peaks at only 3 hours into imbibition. This demonstrates a rapid response to such DNA lesions (Waterworth et al.,

2010). Understanding the link between the molecular controls of repair pathways and seed vigour and viability could further elucidate more molecular determinants of seed quality.

It is possible that further factors that determine seed vigour can be discovered through understanding the molecular basis for DNA repair processes in the *Arabidopsis* seed and building on the work presented in this study. This could facilitate both the eventual development of plant varieties with enhanced germination performance and storability characteristics along with molecular markers for seed quality. DNA repair processes are considered to be highly conserved within higher plants and so work in this study could be readily transferred to agronomically relevant plant species.

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