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A plant DNA ligase is an important determinant of seed longevity

Wanda M. Waterworth[1], Ghzaleh Masnavi[2], Rajni M. Bhardwaj[1], Qing Jiang[2], Clifford M. Bray[2] and Christopher E. West[1,*]

[1]*Centre for Plant Sciences, University of Leeds, Leeds, LS2 9JT, UK, and*

[2]*Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT, UK*

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*For correspondence (fax +44 113 3433144; e-mail c.e.west@leeds.ac.uk).

SUMMARY

DNA repair is important for maintaining genome integrity. In plants, DNA damage accumulated in the embryo of seeds is repaired early in imbibition, and is important for germination performance and seed longevity. An essential step in most repair pathways is the DNA ligase-mediated rejoining of single- and double-strand breaks. Eukaryotes possess multiple DNA ligase enzymes, each having distinct roles in cellular metabolism. Here, we report the characterization of DNA LIGASE VI, which is only found in plant species. The primary structure of this ligase shows a unique N-terminal region that contains a β-CASP motif, which is found in a number of repair proteins, including the DNA double-strand break (DSB) repair factor Artemis. Phenotypic analysis revealed a delay in the germination of *atlig6* mutants compared with wild-type lines, and this delay becomes markedly exacerbated in the presence of the genotoxin menadione. Arabidopsis *atlig6* and *atlig6 atlig4* mutants display significant hypersensitivity to controlled seed ageing, resulting in delayed germination and reduced seed viability relative to wild-type lines. In addition, *atlig6* and *atlig6 atlig4* mutants display increased sensitivity to low-temperature stress, resulting in delayed germination and reduced seedling vigour upon transfer to standard growth conditions. Seeds display a rapid transcriptional DNA DSB response, which is activated in the earliest stages of water imbibition, providing evidence for the accumulation of cytotoxic DSBs in the quiescent seed. These results implicate AtLIG6 and AtLIG4 as major determinants of Arabidopsis seed quality and longevity.

Keywords: DNA damage, repair, DNA ligase, seed, germination, ageing.

INTRODUCTION

Germination is defined as the events that occur in the embryo upon imbibition (water uptake) by the quiescent seed, and which are completed upon emergence of the radicle from the seed coat (Bewley, 1997). Dehydration and rehydration during seed development and germination is associated with high levels of oxidative stress, resulting in DNA damage, including base modification and DNA strand breaks (Dandoy *et al.*, 1987). Genome damage also occurs during seed storage, and is exacerbated by adverse environmental conditions (typically high temperature and relative humidity) that accelerate seed ageing, leading initially to a loss of seed vigour and then to a loss of viability (Cheah and Osborne, 1978). DNA damage must be repaired during imbibition and prior to the initiation of cell division in order to minimize growth inhibition and mutagenesis in subsequent seedling development.

DNA damage usually results in the production of single- and double-strand breaks, either directly through the disruption of the sugar-phosphate backbone of DNA or indirectly through the process of excision repair (Wood, 1996). These breaks are re-joined by the action of DNA ligases. Eukaryotes possess several ATP-dependent DNA ligase activities, each of which have specific roles in cellular DNA metabolism: DNA ligases are essential for the replication and repair of the nuclear and organellar genomes (Bray *et al.*, 2008). Three DNA ligase genes have been identified in mammals, termed *LIG1*, *LIG3* and *LIG4*. DNA ligase I joins Okazaki fragments as part of the DNA replication complex, and *LIG1* genes are essential in humans (*Homo sapiens*), *Saccharomyces cerevisiae* and *Arabidopsis thaliana* (Barnes *et al.*, 1992; Tomkinson *et al.*, 1992; Babiychuk *et al.*, 1998; Taylor *et al.*, 1998a). DNA ligase I has roles in single-strand break (SSB) repair in all eukaryotes, and additional roles in DNA double-strand break (DSB) repair in plants and animals (Liang *et al.*, 2008; Waterworth *et al.*, 2009). *LIG1* also encodes mitochondrial DNA ligase activity in *S. cerevisiae*

and Arabidopsis, with the nuclear and mitochondrial DNA ligase I isoforms arising from alternative translation start sites in a single mRNA transcript, producing DNA ligase protein isoforms either with or without a mitochondrial targeting pre-sequence (Willer *et al.*, 1999; Sunderland *et al.*, 2006).

LIG4 is also present in yeast, mammals and plants (West *et al.*, 2000; Ellenberger and Tomkinson, 2008). DNA ligase IV has specific roles in the non-homologous end joining (NHEJ) pathway of DSB repair (Critchlow *et al.*, 1997) and is important in the survival of Arabidopsis under conditions of genotoxic stress (van Attikum *et al.*, 2003; Friesner and Britt, 2003). NHEJ in plants is mediated by protein complexes of KU70-KU80, LIG4-XRCC4 and the MRE11-RAD50-NBS1 (MRN) complex (Bray and West, 2005). In this pathway DNA rejoining is independent of the DNA sequence, and is the predominant DSB repair mechanism in most plant tissues. Homologous recombination (HR) requires a homologous template for repair, and is catalysed by the RAD52 epistasis group, including the recombinase protein RAD51. DSB detection is linked to intracellular signalling, orchestrated by the protein kinases ATM and ATR, which phosphorylate a number of cellular targets, including the histone variant H2AX (Falck *et al.*, 2005; Friesner *et al.*, 2005).

Mammals possess a third DNA ligase gene, termed *LIG3*, which is expressed as two splice variants (Ellenberger and Tomkinson, 2008). DNA ligase IIIα joins SSBs in DNA, is associated with base excision repair and binds to XRCC1, a protein required for the repair of SSBs induced by γ-irradiation and alkylating reagents (Taylor *et al.*, 1998b). *LIG3β* is expressed only in the testis and may have a role in meiotic recombination (Chen *et al.*, 1995). Human *LIG3* also encodes a mitochondrial form, produced by translation initiation at an alternative upstream translation start site in the *LIG3α* transcript, resulting in a protein with a mitochondrial targeting sequence (Lakshmipathy and Campbell, 2000).

Plants, as sessile photosynthetic organisms, are necessarily exposed to high levels of environmental stresses, including UV-B, gamma irradiation and heavy metals, which have necessitated the evolution of a highly effective DNA damage response to counteract continuous genome damage. Plants require multiple DNA ligase activities to support replicative, repair and recombination activities in the three genomes localized to the nucleus, mitochondrion and chloroplast, leading to the likelihood that multiple DNA ligase genes are present in plants. Analysis of the Arabidopsis genome sequence confirmed this and identified three DNA ligase sequences, two sequences homologous with animal/yeast DNA ligases I and IV, *AtLIG1* and *AtLIG4*, and an uncharacterized DNA ligase, termed *AtLIG6*, which encodes a protein with a domain structure unique to plant species and distinct from those of DNA ligases I, III and IV (West *et al.*, 2000; Molinier *et al.*, 2004; Bonatto *et al.*, 2005; Sunderland *et al.*, 2006; Bray *et al.*, 2008). Here, we report on the functional characterization of DNA LIGASE VI from *A. thaliana* (*AtLIG6*). Our studies reveal that *AtLIG6* is not only required for rapid seed germination under optimal conditions but also becomes an important determinant of germination performance and seed quality in terms of seed viability, vigour and longevity under adverse germination conditions, including accelerated seed ageing, genotoxic stress and low temperature. Additionally, we demonstrate that a rapid and strong DNA DSB response is activated in the earliest stages of seed imbibition, even in high-quality seeds, implicating repair of cytotoxic DSBs accumulated in the quiescent seed as an important integral component of the germination process.

RESULTS AND DISCUSSION

Cloning and sequence analysis of Arabidopsis *LIG6*

The Arabidopsis genomic sequence analysis identified a putative protein with high homology with AtLIG1 (Figure 1). RACE-PCR was used to isolate a full-length cDNA clone [*AtLIG6*; GenBank accession number (gi) AT1G66730] that included an 83-bp 5′-UTR, followed by a 4188-bp open reading frame encoding a putative protein of 1396 amino acids and predicted molecular mass of 156 kDa. The full-length genomic sequence (F4N21_14, gi 12597768, bases 67795–61017, chromosome 1) consisted of 19 exons. The AtLIG6 protein showed a very high similarity with an experimentally validated mRNA from *Ricinus communis* (castor bean), encoding a protein of 57% identity (72% similarity) over a 1336 amino acid overlap with AtLIG6 (Figure 1; RcLIG6; gi 255574286), in addition to predicted proteins in *Oryza sativa* (rice) and *Vitis vinifera* (grape) genomes. No LIG6 homologue was apparent in moss (*Physcomitrella patens*), indicating that higher plants acquired this DNA ligase later in evolution. AtLIG6 also displayed significant sequence similarity to LIG1 proteins, including 38% identity (59% similarity) with AtLIG1 (gi 29029040; At1g08130) over a 641-aa overlap (Figure 1) and 36% identity (56% similarity) with human LIG1 (gi 118773) over a 671-aa region. Bands at the size predicted by analysis of the genomic sequence were obtained in a Southern analysis of Arabidopsis DNA digests, consistent with the genome sequence information indicating that *AtLIG6* is a single copy gene in Arabidopsis (data not presented). The predicted amino acid sequence of AtLIG6 contained a highly conserved DNA ligase catalytic domain towards the C terminus (motif ID PRK01109). The N-terminal region is unique among DNA ligases, and contains a β-lactamase motif found in a large number of proteins (motif ID smart00849; expect value $1e^{-4}$). As previously reported (Molinier *et al.*, 2004; Bonatto *et al.*, 2005), the AtLIG6 β-lactamase motif includes a domain specific to the DNA repair metallo-β-lactamase subset of proteins (β-CASP domain; pfam07522, expect value $3e^{-26}$).

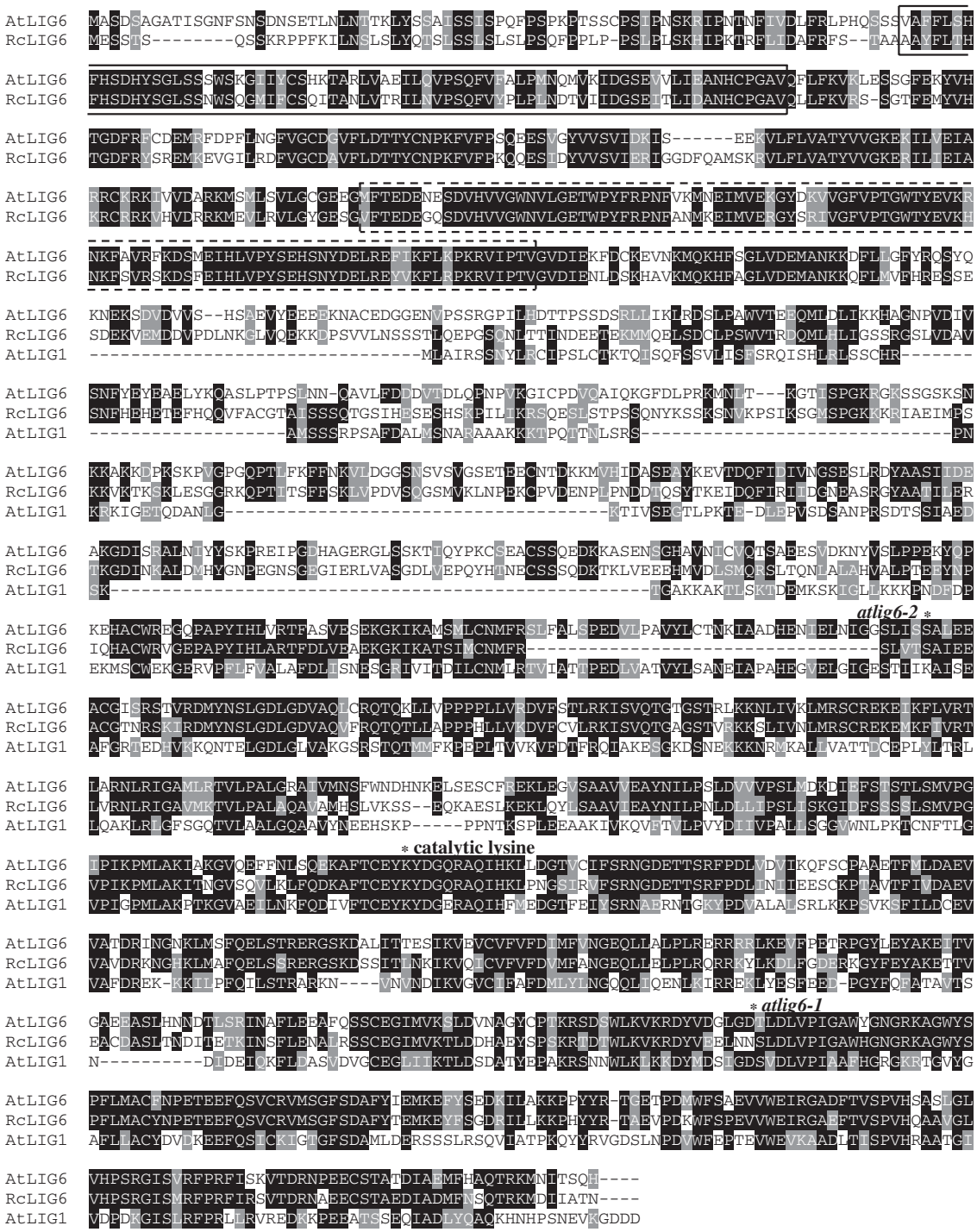

Figure 1. Sequence analysis of AtLIG6.
CLUSTALW sequence alignment of AtLIG6 (AT1G66730) with a predicted LIG6 protein encoded by a sequenced mRNA from *Ricinus communis* (castor bean; gi 255574286) and AtLIG1 (gi 29029040, At1g08130). Aligned residues that are identical are shaded black, and similar residues are shaded grey. The β-lactamase motif (smart00849) is indicated by a solid box, and the β-CASP domain (pfam07522) is indicted by a broken line. The positions corresponding to the mutant lines *atlig6-1* and *atlig6-2* are indicated.

The β-CASP domain was identified in proteins that are involved in nucleic acid processing, including Artemis, PSO2 and the cleavage and polyadenylation specificity factor (Callebaut *et al.*, 2002; Bonatto *et al.*, 2005). Yeast and mammalian PSO2 proteins are involved in interstrand DNA crosslink repair, wherea Artemis functions in V(D)J

recombination, and NHEJ is required for processing complex DNA ends prior to end joining, including hairpin structures (Bonatto *et al.*, 2005). Interestingly, in the thermophilic Archaea *Acidianus ambivalens* a β-lactamase domain-encoding gene is located in the same cistron immediately 3′ to an ATP-dependent DNA ligase gene (gi 40784), suggesting the conserved association of the DNA ligase and β-lactamase motifs in divergent taxa. The presence of the β-CASP domain is consistent with a specialized role for AtLIG6 in pathways of interstrand crosslink or DSB repair in higher plants.

Analysis of *AtLIG6* expression patterns

AtLIG6 expression was investigated by an analysis of publically available microarray data and real-time RT-PCR (Figure 2). *AtLIG6* was expressed at low levels in all tissues investigated, but exhibited elevated expression in flowering tissues and meristems (Figure 2a). Expression in vegetative tissues indicates that the role of AtLIG6 is not restricted to meiotic recombination, and that AtLIG6 is likely to be involved in DNA repair and/or DNA replication processes. The highest level of *AtLIG6* expression in all array studies was found in dormant seeds following prolonged imbibition of up to 30 days at 20°C (Cadman *et al.*, 2006) (Figure 2b). The seeds of Arabidopsis ecotype Cvi (gi N8580) acquire primary dormancy during development, and require both light and nitrate to germinate. This dormancy is lost during 120 days of after-ripening (Cadman *et al.*, 2006). The high levels of *AtLIG6* expression in dormant hydrated seeds (Figure 2b) suggest physiological roles for AtLIG6 in genome repair during seed imbibition. Imbibing Col-0 seeds also expressed both AtLIG4 and AtLIG6, raising the possibility that these ligases have roles in germination. Unlike Cvi seeds, Col-0 seeds are not naturally dormant. However, treatment of Col-0 with abscisic acid (1 mM) to prevent germination and simulate the dormancy of Cvi did not result in the upregulation of the *AtLIG6* transcript (data not shown). The roles of DNA ligase in genome repair during imbibition was further investigated by analysis of *AtLIG6*- and *AtLIG4*-deficient seeds.

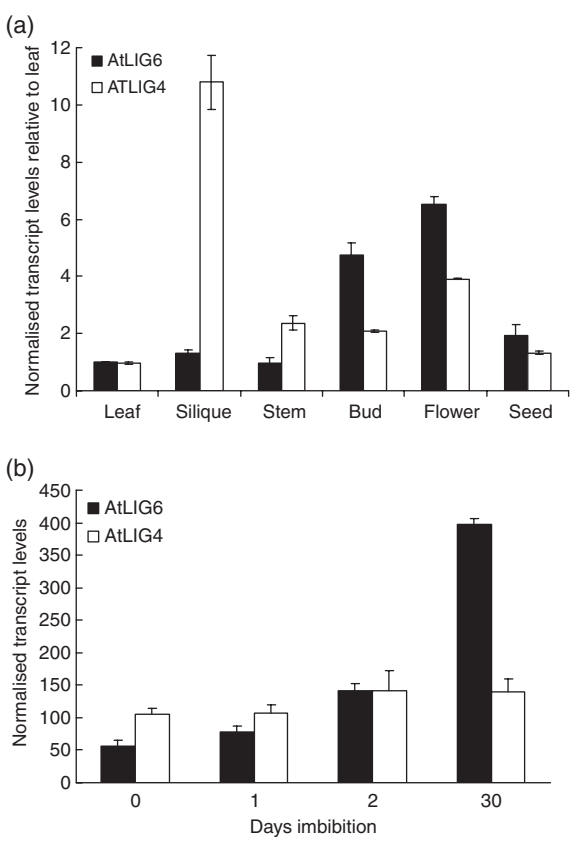

Figure 2. Analysis of *AtLIG6* expression patterns.
(a) Real-time PCR analysis of *AtLIG4* and *AtLIG6* expression in Arabidopsis tissues (Col-0) including leaf, silique, stem, flower, bud and seeds at 1 h of imbibition. cDNA levels were normalized to *ACTIN2*.
(b) Expression profile of *atlig6* in dormant Cvi seeds imbibed for the indicated period, determined by microarray analysis as described by Cadman *et al.* (2006).

Isolation of insertional knock-out mutants of *AtLIG6* in *Arabidopsis thaliana*

Mutants in the *AtLIG6* gene were identified in the collection of sequence-tagged T-DNA mutants (http://signal.salk.edu/cgi-bin/tdnaexpress). Two alleles were characterized: *atlig6-1* was found in the SALK collection (Alonso *et al.*, 2003) and *atlig6-2* was found in the INRA-Versailles collection (Samson *et al.*, 2002). The T-DNA insertion in *atlig6-1* (SALK_079499) was located in exon 14, corresponding to the DNA ligase core catalytic domain, which would be expected to disrupt DNA ligase function (Figure 3a). Line *atlig6-2* (FLAG_437H07) carries a T-DNA insertion in exon 4 of *AtLIG6*. This corresponds to the region between the β-CASP domain and the DNA ligase domain of the AtLIG6 protein sequence (Figure 3a). The T-DNA left border–*AtLIG6* junction was complex in the *atlig6-2* line, with an insertion derived from the left border sequence, including three copies of an 11-bp sequence (underlined in Figure 3a). RT-PCR analysis confirmed that *AtLIG6* transcript was undetectable in both lines (Figure 3b). In addition, an *atlig4* mutant was also isolated from the SALK collection, termed *atlig4-5* (SALK_095962), and contained a T-DNA insertion in exon 10 (Figure 3c). The *atlig4-5* allele displays the same phenotype as the *atlig4* alleles published previously (van Attikum *et al.*, 2003; Friesner and Britt, 2003; Kozak *et al.*, 2009) (Figure 4e).

Characterization of *atlig6* mutant plants and *atlig4-5 atlig6-1* double mutants

Both *atlig6* mutant alleles displayed normal 3:1 segregation patterns, indicating that AtLIG6 is not essential for microspore development, and that homozygous mutants are viable (data not presented). The mutant lines displayed normal vegetative growth, illustrating that AtLIG6 is not essential for

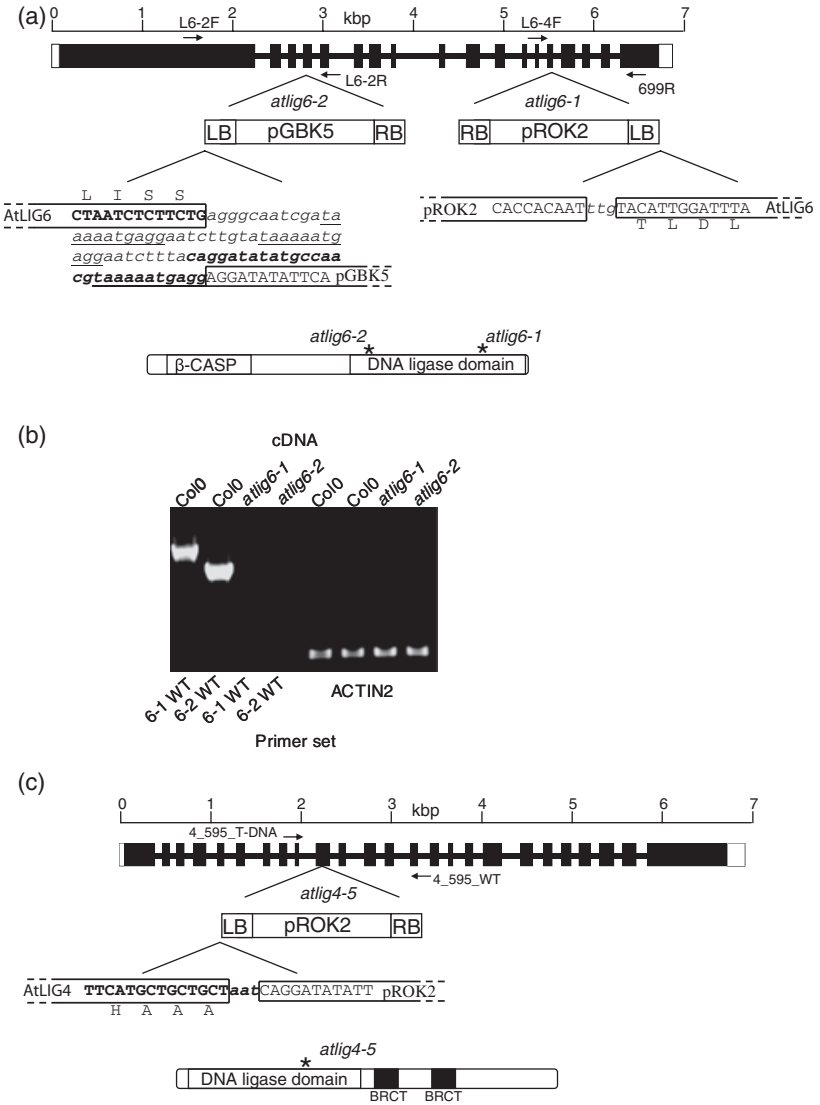

Figure 3. Isolation of *atlig6* and *atlig4* mutant lines.
(a) Schematic of the *AtLIG6* gene. Exons are denoted by boxes, with empty boxes representing the untranslated regions and filled boxes representing the coding regions, and with introns represented by a line. The position of T-DNA insertions in *atlig6-1* and *atlig6-2* lines are indicated with the sequence of the T-DNA left border–*AtLIG6* genomic junction shown. The region in the AtLIG6 protein corresponding to the T-DNA insertion is indicated by an asterisk.
(b) RT-PCR analysis demonstrating the absence of full-length *AtLIG6* transcript in the *atlig6-1* and *atlig6-2* mutant lines. Amplification of *ACTIN2* provides a control for cDNA synthesis.
(c) Schematic of the *AtLIG4* gene, as described for *atlig6* above.

the replication of nuclear or organellar genomes (Figure 4a,b). Null *atlig6* mutants were fully fertile, demonstrating that the resolution of recombination intermediates and repair of SPO11-induced DNA breaks occurs in the absence of AtLIG6 activity (Figure 4c,d). To investigate the possibility that AtLIG4 compensates for the absence of AtLIG6 in organellar genome replication or in meiosis, *atlig4-5 atlig6-1* double mutants were isolated and shown to be phenotypically indistinguishable from wild-type plants under normal growth conditions (Figure 4a–d). These results suggest that chloroplast genome replication is mediated by AtLIG1, or an as yet unidentified DNA ligase activity in Arabidopsis, and that AtLIG4 or AtLIG6 activities are not essential for the completion of meiosis. Equivalent numbers of seeds in siliques of wild-type and mutant lines also indicates that *atlig6-1* and *atlig4-5 atlig6-1* lines are not defective in seed development (Figure 4c).

Phenotypic analysis of *atlig6* mutants

Despite extensive testing with a range of DNA damaging agents (UV-C, methyl methanesulfonate, mitomycin C, menadione and bleomycin), the only significant growth hypersensitivity of the *atlig6* mutant lines was identified after a 100-Gy dose of X-rays (Figure 4e). This resulted in root growth that was 77% ($P < 0.05$) that of similarly treated Col-0 mutant seedlings 10 days after irradiation, indicating functions of AtLIG6 in the repair of X-ray-induced damage. In contrast, 100 Gy is lethal to *atlig4-5* mutants, with root growth stopping after germination (Figure 4e). This low hypersensitivity of the *atlig6* lines suggests that either AtLIG6 does not have a major function in DNA repair or that AtLIG6 activity is redundant to another cellular ligase activity. Alternatively, the DNA repair activity of AtLIG6 may be highly specific to a DNA lesion not induced by most of

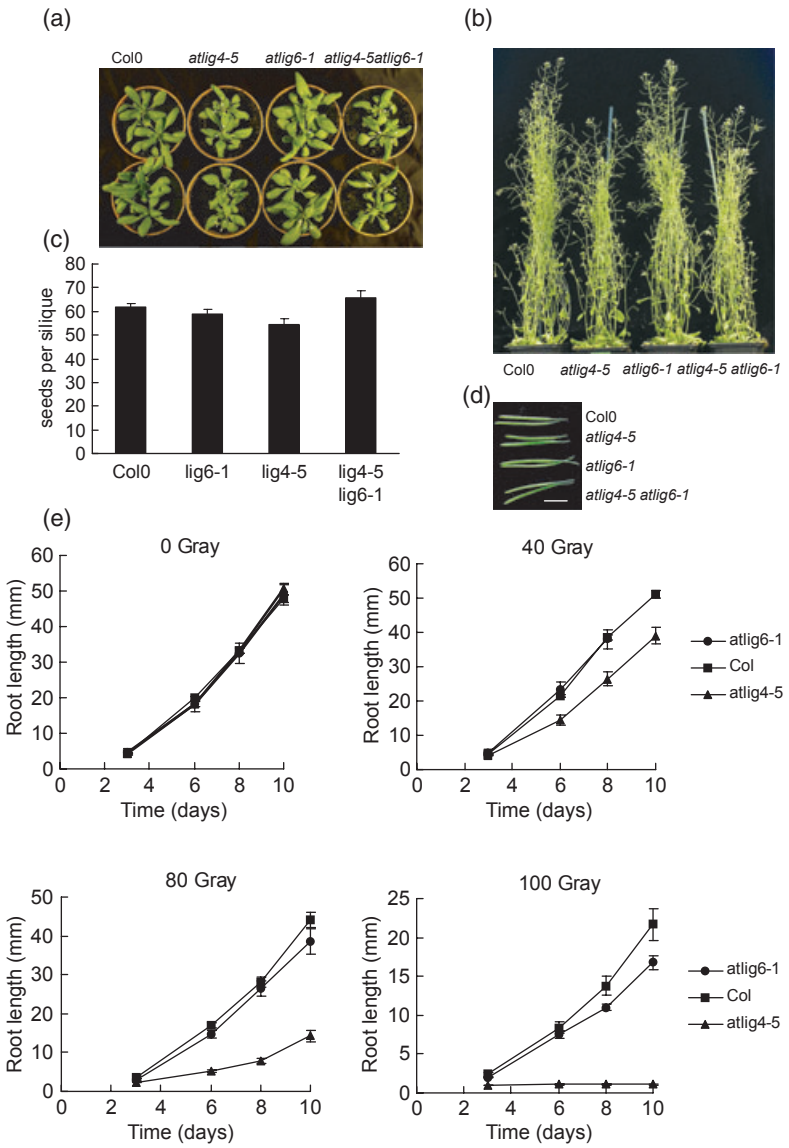

Figure 4. Phenotypic analyses of *atlig6-1* and *atlig6-1 atlig4-5* mutant lines.
(a) Homozygous *atlig6-1* and *atlig4-5 atlig6-1* double mutant lines are viable, and display normal vegetative growth.
(b) *atlig6-1* and *atlig4-5 atlig6-1* produce flowers with viable pollen and ovules.
(c) Seed production in the *atlig6-1*, *atlig4-5* and *atlig4-5 atlig6-1* lines is not significantly different to that of wild-type lines ($P > 0.05$).
(d) Silique morphology is similar in the wild type and *atlig6-1* and *atlig4-5 atlig6-1* lines, consistent with wild-type levels of seed production.
(e) Root growth of Col-0, *atlig6-1* and *atlig4-5* lines after exposure to irradiation with X-rays at the indicated dose. At 100 Gy, *atlig6-1* plants display a significant reduction in root growth relative to wild-type lines ($P < 0.05$). Error bars are the standard error of the mean.

the treatments tested, or to particular developmental stages when alternate ligase activities cannot compensate for *atlig6* deficiency. The potential redundancy of AtLIG6 as a result of AtLIG1 (which participates in nuclear and mitochondrial genome maintenance, with SSB and DSB repair activities in plants; Taylor *et al.*, 1998a; Waterworth *et al.*, 2009) was difficult to investigate, because *atlig1* knock-out lines are lethal (Babiychuk *et al.*, 1998). However, the expression of *AtLIG6* in seeds prompted a detailed phenotypic analysis of germination performance of the *atlig6* mutant lines.

Germination analysis of DNA ligase mutants

Dehydration and rehydration during seed development and imbibition is associated with high levels of DNA damage (Dandoy *et al.*, 1987). Genome damage also occurs during seed storage, and loss of seed viability correlates with the accumulation of DNA breaks and chromosome rearrangements, clearly establishing a link between DNA damage incurred during seed ageing and reduced germination potential (Roberts, 1972; Cheah and Osborne, 1978; Osborne, 1982). Repair of accumulated DNA damage is initiated in the earliest stages of imbibition, observed as high levels of unscheduled *de novo* DNA synthesis in seeds several hours before activation of the cell cycle and entry into the S phase (Osborne *et al.*, 1984). Higher levels of repair synthesis occur in aged seeds, often accompanied by a delay in the onset of replicative DNA synthesis and delayed germination or loss of viability (Osborne *et al.*, 1984). The expression of *AtLIG6* observed in Cvi and Col-0 seeds led to the hypothesis that DNA repair activities also occur in the dormant, imbibed seeds, as previously

observed in *Lactuca sativa* (lettuce) seeds (Villiers, 1974). Pre-sowing treatments such as seed priming improve seed germination performance and have been shown to be advantageous in producing early and uniform seed germination and seedling emergence, with benefit to seedling establishment in the field. One such priming treatment termed osmopriming (Heydecker *et al.*, 1975) involves controlled hydration of seeds to bring them to the brink of germination, whilst preventing their entry into the final phase of germination (Karssen *et al.*, 1990). Significant levels of nuclear DNA repair synthesis in the absence of any detectable nuclear DNA replication or cell division has been demonstrated in the embryos of both high- and low-vigour *Allium porrum* L. (leek) seeds during osmopriming, consistent with the importance of DNA repair processes to germination performance (Ashraf and Bray, 1993). However, prolonged imbibition of seeds in water under conditions unfavourable for germination may result in the extended exposure of the seed embryo genome to attack by reactive oxygen species (ROS) generated upon water uptake, leading to a requirement for DNA ligase-mediated DNA repair.

Germination analysis of high quality (unaged) wild-type seeds and *atlig6* mutant seeds revealed delayed germination in the AtLIG6-deficient lines. Radicle emergence was delayed by 6 h in seeds of *atlig6-1* mutants compared with wild-type Col-0 seeds; *atlig6-1* seeds reached 50% germination after 35 h of imbibition, compared with wild-type Col-0 lines, which attained 50% germination by 29 h of imbibition (Figure 5a). These results indicate that even in the absence of environmental conditions that adversely affect seed germination performance, AtLIG6-mediated DNA repair pathways are still necessary to facilitate rapid germination. However, there was no significant difference in final seed viability scores between mutant and wild-type lines, with all lines studied displaying near 100% germination after 48 h (Figure 5a). Comparable results were obtained with both *atlig6* alleles (*atlig6-1* and *atlig6-2*), suggesting that the AtLIG6 has roles in repairing DNA damage accumulated during seed development, storage and/or imbibition (Figure 5a,b). In contrast with previously published data (Friesner and Britt, 2003), *atlig4* mutants also displayed a delay in germination relative to wild-type lines, with reproducible delays observed in two independent alleles *atlig4-5 and atlig4-2* (Figure 5a,b), which may reflect differences in germination or growth and seed harvest/storage conditions between the two studies. The *atlig4-5 atlig6-1* double mutant displayed a similar germination rate to the *atlig6-1* line (Figure 5a). DNA ligases are likely to be required for the repair of DNA damage that accumulates in seeds, which correlates with a loss of seed viability (Cheah and Osborne, 1978), and is also present in high-quality seeds resulting in DNA repair synthesis early in imbibition (Ashraf and Bray, 1993).

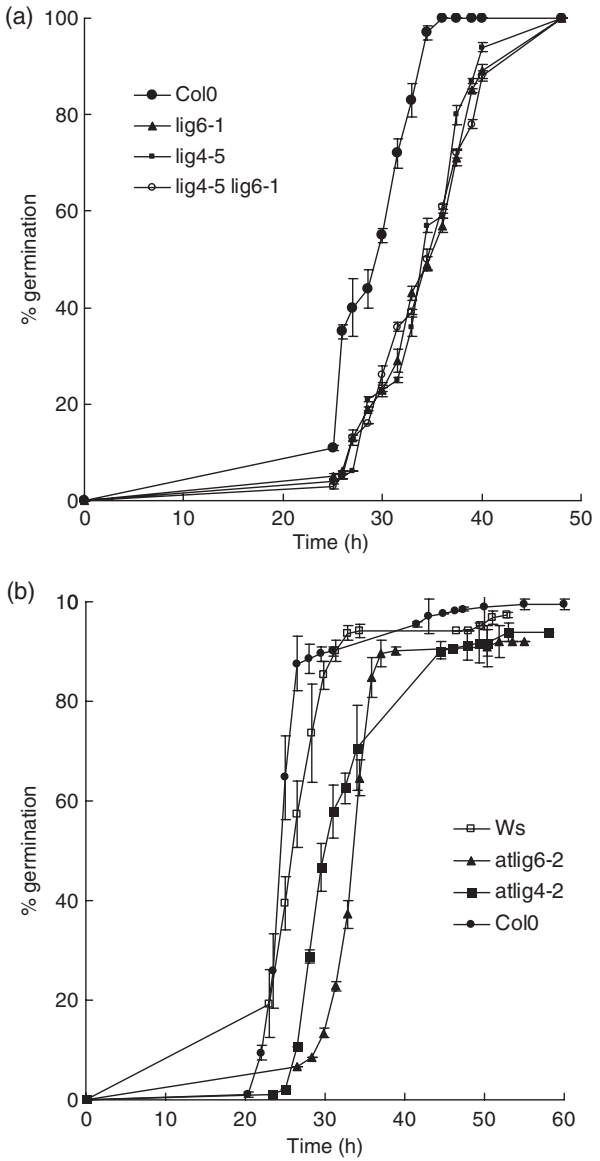

Figure 5. Germination performance of *atlig6* and *atlig4* mutants.
Germination performance at 20°C under light. Seeds were plated onto MS plates and stratified at 4°C for 48 h before transfer to 20°C.
(a) Percentage germination of Col-0, *atlig6-1*, *atlig4-5* and *atlig4-5 atlig6-1* double mutant.
(b) Percentage germination of Ws, Col-0, *atlig4-2* and *atlig6-2*. Values are from three replicates of 100 seeds, with the standard error of the mean indicated.

DNA ligases are important for seed longevity

The delayed germination exhibited by *atlig6* mutants suggests that DNA damage and repair pathways are important to seed germination and seed quality. Seed longevity or storability is an important agronomic aspect of seed quality, and is also of importance for the conservation of plant genetic resources. Increases in seed moisture content and temperature during storage result in quantifiable and

predictable decreases in seed viability (Roberts, 1973). Cytological studies, quantifying aberrant products of DSB repair in seeds, concluded that even small losses of seed viability through ageing are inevitably associated with an increase of chromosome damage (Dourado and Roberts, 1984). The 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 damage to the genome. This is analogous with the effects of genotoxic agents or deficiencies in repair capacity that slow germination. Therefore, the response of *atlig6-1*, *atlig4-5* and *atlig4-5 atlig6-1* mutants to accelerated ageing was investigated. Seeds of Arabidopsis wild type and mutant lines were aged as previously described (Hay *et al*., 2003) at 45°C over KCl to a relative seed moisture content of 10.8% for 8 days. Seeds were either plated directly on MS plates or stored at 2°C prior to plating. This ageing regime resulted in delayed radicle emergence in the wild-type seeds without affecting viability, but greatly reduced seed viability and vigour in the *atlig4-5*, *atlig6-1* and *atlig4-5 atlig6-1* double mutants (Figure 6a). At 8 days almost 100% of the wild-type seeds had germinated, as opposed to around 50% of *atlig6-1* and 75% of *atlig4-5* (Figure 6a). Strikingly, almost no *atlig4-5 atlig6-1* seeds germinated at 8 days imbibition. Germination scores were continued up to day 26, after which no further seeds germinated, with final germination percentages of 93% for wild type, 80% for *atlig4-5*, 70% for *atlig6-1* and 40% for *atlig4-5 atlig6-1*. This indicates that deficiencies in AtLIG4 and AtLIG6 result in both decreased seed viability and seed germination vigour with seed ageing. That the most detrimental effects on seed viability were in *atlig4-5 atlig6-1* double mutants suggests that either AtLIG4 and AtLIG6 function in distinct pathways with different specificities for DNA lesions, or that both enzymes are needed in pathways to remove the levels of DNA damage encountered in aged seeds. Measurements of root length in germinated seedlings from each seed lot at 8 and 10 days show substantially reduced seedling vigour in the mutant lines (Figure 6b,c). These results indicate that both NHEJ (involving AtLIG4) and also an uncharacterized repair pathway (involving AtLIG6) are important contributors to seed longevity. These results implicate the capacity of the embryo to repair DNA damage that accumulates during storage as being a determinant of seed resilience to deterioration during storage, and the subsequent germination vigour of stored seed lots. Thus, although AtLIG6 facilitates germination in unstressed seeds, it becomes an increasingly important determinant of the quality of aged seeds.

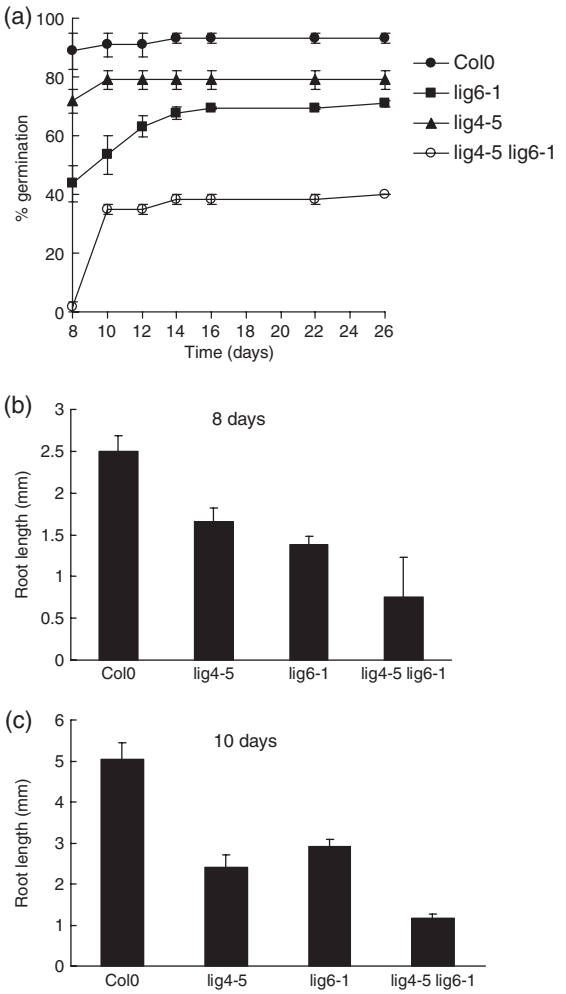

Figure 6. Analysis of *atlig6-1, atlig4-5* and *atlig4-5 atlig6-1* germination performance and seedling vigour after ageing treatment.
Seed viability and vigour of the wild type and DNA ligase mutant lines after controlled ageing. Seeds were aged at 45°C for 8 days, with a relative moisture content of 10.8%, and placed at 4°C for 48 h before transfer to an environmental growth chamber at 20°C.
(a) Percentage of seeds germinated each day post-imbibition.
(b) Mean root length of germinated seeds at 8 days post-imbibition.
(c) Mean root length of germinated seeds at 10 days post-imbibition. Error bars represent the standard error of the mean. Values from three replicates of 100 seeds.

DNA ligase 6 is important for germination vigour under cold temperature stress

The germination performance of wild type and DNA ligase-deficient lines was evaluated under suboptimal conditions using low temperature stress (germination at 2°C). Germination at low temperatures substantially slows radicle emergence, and is a stress that can be encountered by field-grown crops sown in early spring and late autumn. We analysed seed quality by measuring germination rates, whereas seedling vigour was assessed by the measurement of root elongation rates. After germination at 2°C for 14 days, wild-type seeds displayed high levels of germination (82%), whereas only 52% of *atlig4-5* mutants germinated, as scored by radicle emergence (Figure 7a). In contrast, although the seed coat had cracked on some

atlig6-1 and *atlig4-5 atlig6-1* seeds, there was no sign of radicle protrusion. Seeds were then transferred to standard growth conditions to evaluate the effects of suboptimal germination conditions on subsequent seedling vigour. By 4 days most remaining seeds from the mutant lines germinated (90–98%), indicating that low temperature stress influences germination vigour as opposed to seed viability *per se* (data not shown). As seeds were exposed to light throughout these treatments, the delayed germination of *atlig6-1* and *atlig4-5 atlig6-1* seeds at low temperatures is likely to represent a response to the stress rather than the appearance of secondary dormancy. Root length measurements showed that germination under cold stress significantly affected subsequent seedling vigour in mutant lines (Figure 7b). Low temperature stress primarily results in a slowing of the germination processes, although prolonged imbibition of seeds at low temperature may act as an additive source of DNA damage because of increased oxidative stress.

DNA ligase 6 is important for germination under oxidative stress

Previous analysis of *atlig4-1* mutants had identified roles for AtLIG4 in the germination of seeds subjected to irradiation by X-rays (Friesner and Britt, 2003). Oxidative damage generated by ROS is believed to be the major source of DNA damage in germination under normal growth conditions (Bray and West, 2005). Germination of seeds imbibed in the presence of the genotoxin menadione, which induces oxidative damage to DNA, was used to examine the roles of AtLIG6 in germination under conditions of genotoxic stress. Seeds were imbibed at 4°C for 24 h in the presence of menadione in order to ensure the uptake of the toxin prior to the onset of germination. Wild-type Arabidopsis seeds displayed a progressively delayed time to radicle emergence (51% germination at 4 days in 50 μM menadione) and decline in seed viability to 52% (Figure 7c). The results presented in Figure 7(c) indicate that the delayed germination of the *atlig6-1* and *atlig6-2* lines is exacerbated in the presence of menadione, with the mutant lines taking 3.5–5.5 days longer to reach 25% germination. This reduced vigour in the presence of menadione indicates that the AtLIG6-dependent DNA repair pathway is important for germination in the presence of genotoxic stress (Figure 7c), and emphasizes the relationship between DNA damage, repair capacity and timing of germination. The genetic interaction between *AtLIG4* and *AtLIG6* is unclear. The additive nature of the mutations is consistent with partial redundancy between the ligases (Figure 6), whereas Figure 4 clearly displays the major role of *AtLIG4* in the repair of X-ray-induced DNA damage, with only a minor role for *AtLIG6*. This may reflect difference in substrate specificity between the ligases, or differences in activity between tissue types.

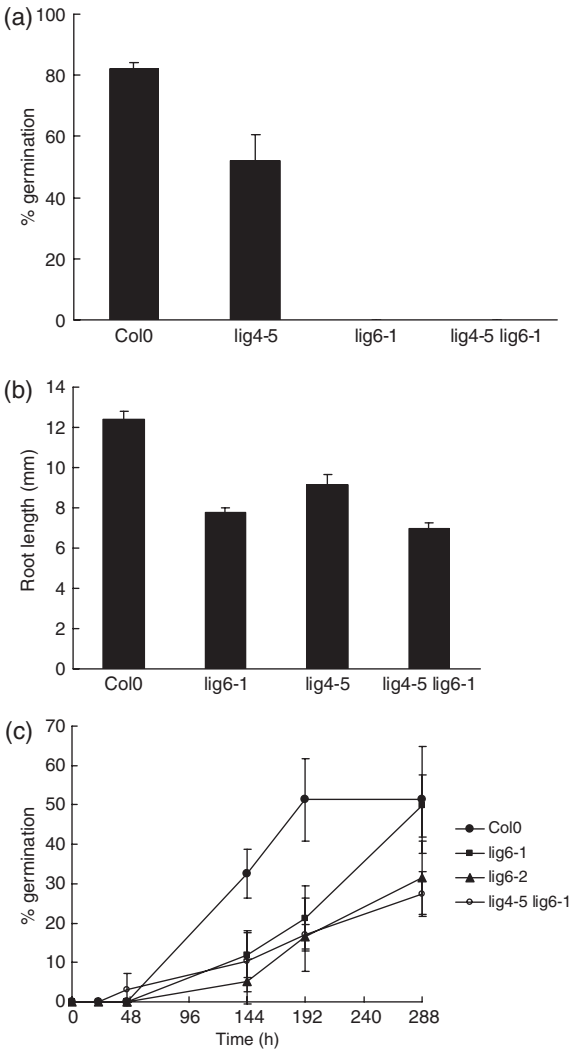

Figure 7. Analysis of *atlig6-1*, *atlig4-5* and *atlig4-5 atlig6-1* germination performance and seedling vigour after low temperature germination.
(a) Seeds were placed on MS plates held at 2°C for 14 days, and the percentage of germinated seeds was then counted.
(b) Mean root length of germinated seedlings following 10 days at 20°C subsequent to 14 days held at 2°C. Error bars represent the standard error of the mean. Values from three replicates of 100 seed.
(c) Sensitivity of *atlig6-1* and *atlig6-2* germination performance to the presence of menadione. Seeds were imbibed at 4°C in the presence of 50 μM menadione for 48 h before plating onto MS plates and transfer to standard germination conditions (20°C) in a controlled environment chamber. Germination was scored daily. Values are from three replicates of 100 seeds each.

Evidence of DNA damage in germinating seeds

The results presented in Figures 5–7 demonstrate roles for both AtLIG6 and AtLIG4 in germination. This requirement for DNA repair pathways for rapid germination suggests that even in high-quality seeds that have been harvested, stored and germinated under optimal conditions, DNA damage still accumulates and needs to be repaired during imbibition.

To investigate the appearance of DSBs in non-aged seeds, we examined the DNA damage response in imbibing seeds using an analysis of publically available microarray data (Nakabayashi *et al.*, 2005; Winter *et al.*, 2007). When exposed to clastogens, plants display a very well-characterized, ATM-dependent transcriptional induction, specifically in response to the presence of DSBs (Culligan *et al.*, 2006; Ricaud *et al.*, 2007). Microarray analysis of temporal gene expression during Arabidopsis seed germination identified coordinated upregulation of a number of DSB-responsive genes in the earliest stages of imbibition, coincident with the reported incidence of DNA repair synthesis (Nakabayashi *et al.*, 2005). This DSB-induced DNA damage response resulted in the significant upregulation of transcripts within 3 h of imbibition (Figure 8a), including RAD51 and PARP2, a protein that plays a number of roles in the DNA damage response. RAD51 expression patterns were verified by semi-quantitative RT-PCR and real-time PCR, confirming the transcriptomics data (Figure 8b,c). In contrast, *AtLIG4* and *AtLIG6* transcripts showed no significant transcriptional upregulation. Nuclear DNA replication occurs later than DNA repair, around the time of radical emergence (Barroco *et al.*, 2005). In Arabidopsis, it is likely that the S phase and cell division precede radical emergence, as cell cycle activity is required for rapid germination (Masubelele *et al.*, 2005). The repair of these lesions is an important aspect of germination, and provides an explanation for the accumulation of chromosomal aberrations associated with a loss of viability during seed ageing (Dourado and Roberts, 1984). Interestingly, the DNA damage response, indicated by induction of the *AtRAD51* transcript, was significantly higher in both the *atlig4* and *atlig6* mutant lines, compared with Col-0. This suggests that deficiency in either DNA ligase leads to an accumulation of DNA damage in seeds, and is consistent with the observed delay in germination in both mutant lines (Figure 5). Previous studies of *atku80* mutants revealed a constitutive upregulation of DNA damage in seedlings in the absence of external stresses (West *et al.*, 2004), and hypersensitivity to methyl methanesulfonate-induced DNA damage specifically during germination (Riha *et al.*, 2002).

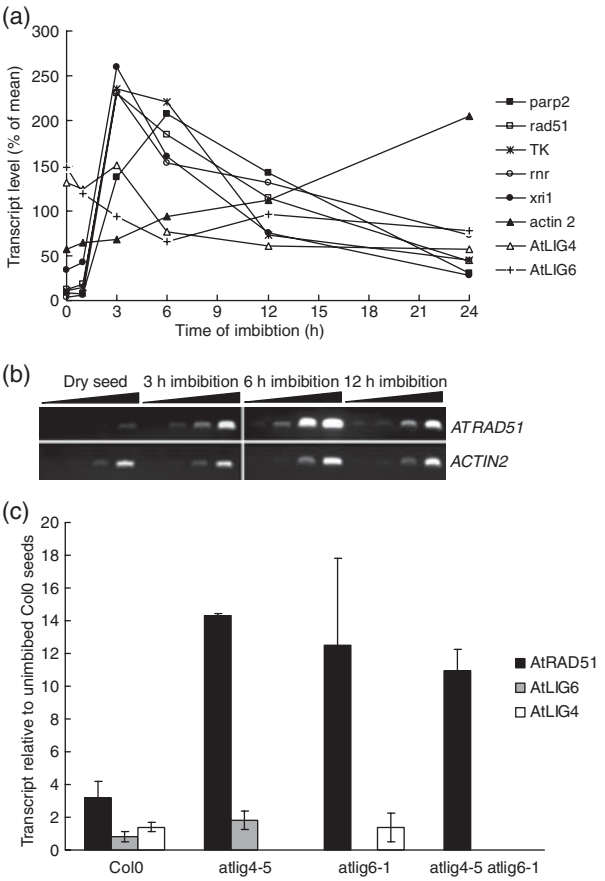

Figure 8. Activation of the double-strand break (DSB) transcriptional response in imbibed seeds.
(a) Transient transcriptional activation of DSB-responsive genes at 0–24 h imbibition (normalized microarray data); the *ACTIN2* control is indicated; *PARP2*, AT4G02390; *RAD51*, AT5G20850; *THYMIDINE KINASE*, AT3G07800; *RIBONUCLEOTIDE REDUCTASE* small subunit/*TSO2*, AT3G27060; *XRI1*, AT5G48720; *ACTIN2*, AT3G18780; *AtLIG4* and *AtLIG6*.
(b) RT-PCR confirmation of array data for *AtRAD51* expression showing 1:10 serial dilutions of cDNA synthesized from RNA isolated from imbibing seeds. Control RT-PCR was performed using primers specific to *ACTIN2*.
(c) Real-time PCR showing transcript levels after 6 h of imbibition in mutant and wild-type lines, compared with those of unimbibed Col-0 seeds. AtRAD51 transcripts are induced, particularly in the DNA ligase mutant lines.

DNA repair mechanisms required for germination in the presence of DNA damage

In plants, the accumulation of DNA strand breaks during storage of orthodox seeds (i.e. seeds that survive drying to low moisture content) reduces viability and vigour, although the specific DNA lesions responsible for impaired germination and their repair pathways remain poorly defined. Recent studies point to a role for the DNA damage response protein poly(ADP-ribose) polymerase (PARP) in germination (Hunt *et al.*, 2007), although the loss of base excision repair factors did not affect germination (Murphy and George, 2005), suggestive that in plants, as in other organisms, high levels of base damage can be tolerated. Following irradiation of seeds, the Arabidopsis mutants *atku70* and *atlig4* displayed delayed germination relative to irradiated wild-type controls, supportive of roles for NHEJ in the repair of DNA DSBs as a necessary prerequisite for the germination of irradiated seeds (Friesner and Britt, 2003). Phenotypic analysis of irradiated kernels of maize *rad51* (an HR pathway component) mutant lines also revealed a severe delay in seed germination relative to wild-type lines (Li *et al.*, 2008). The results of the present study clearly demonstrate that the accumulation of DNA DSBs and the capacity to repair these DSBs influences the germination rate of seeds. This indicates that genome integrity is sensed during imbibition and is part of the control mechanism that determines the timing of

germination, as measured by radical emergence. In Arabidopsis, a resumption of cell cycle activity in the root is required for germination (Masubelele *et al.*, 2005), with important roles for the cyclins CYCD4;1 and CYCD1;1. In particular, Arabidopsis *cycd1;1* mutants display greatly delayed radicle emergence, with an approximate 80–90% reduction in germination at the 24-h time point, which correlates with delayed cell division in the embryo root apex. Thus, the detection and signalling of DNA damage in aged or mutant seeds may limit mitotic activation, possibly through the WEE1-mediated G2/M checkpoint (De Schutter *et al.*, 2007), or may activate a G1/S checkpoint as observed in irradiated wild-type and *atlig4* seedlings (Hefner *et al.*, 2006).

CONCLUSIONS

In all organisms genomic integrity is maintained by a sophisticated array of specific DNA repair pathways evolved to repair the spectrum of lesions induced in the genome by genotoxic stresses, including base damage, SSBs and DSBs. The well-established correlation of DNA damage with seed ageing suggests that DNA repair pathways, including those for repair of cytotoxic DSBs, play an integral role in seed germination and in the longevity of stored seeds. However, the specific genes involved in these DNA processes remain to be identified. Here, phenotypic analysis of *atlig6* mutant plants illustrates important roles for this DNA ligase in seed germination and seed longevity, in particular under genotoxic stress conditions. Similarly, the delay in germination exhibited by *atlig4* mutants and the rapid DNA damage transcriptional response identified in imbibing seeds implicate DSBs and their associated repair pathways as factors that influence seed germination performance. In particular, we identify significant roles for DNA ligases in seed germination, in terms of vigour and viability, following storage under suboptimal conditions, as encountered in much of the developing world. In addition, the prolonged storage in seed banks as part of plant germplasm conservation programmes relies on the ability to maintain viability to allow the subsequent propagation of archived species. The elucidation of the DNA repair mechanisms that are important for rapid germination and seed longevity will help predict storage and germination performance of seed lots, and these DNA repair pathways represent targets for the generation of crops with improved seed storability/germination performance characteristics.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis (Col-0) plants were raised in growth chambers (Sanyo, http://www.sanyo-biomedical.co.uk/index.htm) or glasshouses under controlled conditions of constant humidity (30%), with 16-h light/8-h dark cycles at 23°C. Arabidopsis mutants were identified using the T-DNA express genome browser (http://signal.salk.edu/cgi-bin/tdnaexpress) and ordered through the Nottingham Arabidopsis Stock Centre(NASC, http://arabidopsis.info) (Scholl *et al.*, 2000): *atlig6-1* (SALK_079499); *atlig6-2* (FLAG_437H07); *atlig4-5* (SALK_095962); *atlig4-2* (SAIL_597_D10). The following oligonucleotide primers were used for plant genotyping: *atlig4-5*, 4_595_WT, 5′-AAAGCCCTAAGGTCTTCATGG-3′, 4_595_T-DNA, 5′-TTTGTTGTTTGAGGATCCGAC-3′; *atlig6-1*, L6_4F, 5′-GTTGGCTCTTCCCCTCCGTGAGAGAC-3′, 699_T-DNA, 5′-GCAAG-GATCTTATCCTCCGAG-3′ and the T-DNA specific primer SALK_LBb1, 5′-GCGTGGACCGCTTGCTGCAACT-3′; *atlig6-2* was screened using L6-2F, 5′-GGTGGAGAGAATGTTCCATCATCTAGAG-3′, L6-2R, 5′-GTCGACAAACGCAAAAGCTACTGGTTCCTC-3′ and the T-DNA specific primer INRA-LB4, 5′-CGTGTGCCAGGTGCCCACGGAATAGT-3′.

Bioinformatic analysis

Protein sequence alignment was performed using ClustalW (Chenna *et al.*, 2003) and annotated using boxshade (http://www.ch.embnet.org/software/BOX_form.html). Protein motifs were identified through NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Microarray analysis was performed using the eFP browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter *et al.*, 2007) using data deposited by Cadman *et al.* and Nakabayashi *et al.* The experiments by Cadman *et al.* (2006) analysed transcript levels in dormant, unstratified Cvi seeds imbibed for 1, 2 or 30 days at 20°C in the dark on water-moistened filter paper. Microarray analysis used ATH1 Affymetrix arrays, performed by the NASC service. In the study of Nakabayashi *et al.* (2005) Col-0 seeds were imbibed on filter paper moistened with water under light at 22°C, after a washing step, but without prior stratification. Transcripts were analysed using ATH1 arrays and data were normalized in GeneSpring (http://www.agilent.com/chem/genespring). The accession numbers for the genes analysed are: *AtLIG1*, AT1G08130; *AtLIG4*, AT1G49250; *AtLIG6*, AT1G66730; *HsLIG1*, gi 118773; *Ricinus communis*, gi 255574286. Raw array hybridization scores were normalized to give a percentage score based on the mean value of expression in the array experiments analysed for the following genes: *PARP2* (AT4G02390); *RAD51* (AT5G20850); *THYMIDINE KINASE* (AT3G07800); *RIBONUCLEOTIDE REDUCTASE* small subunit/*TSO2* (AT3G27060); *XRI1* (AT5G48720); *ACTIN2* (AT3G18780).

Molecular cloning

DNA procedures and bacterial manipulations were performed using established protocols (Sambrook *et al.*, 1989), unless otherwise stated. Plasmid DNA was prepared on an analytical scale using QIAGEN columns according to the manufacturer's instructions (Qiagen, http://www.qiagen.com), and RNA was isolated from plant tissues using the SV total RNA isolation kit (Promega, http://www.promega.com).

AtLIG6 was cloned by RT-PCR. cDNA synthesis was performed with AMV reverse transcriptase (Roche, http://www.roche.com) and followed by amplification with iProof DNA polymerase (Bio-Rad, http://www.bio-rad.com). RACE-PCR was performed using the 3′/5′ RACE-PCR kit (Roche) according to the manufacturer's instructions. PCR products were cloned using a TOPO-TA cloning kit (Invitrogen, http://www.invitrogen.com), following incubation of the purified PCR products with Taq. Analytical PCR was performed using PCR Reddymix (ABGene, http://www.abgene.com) and analysed by agarose gel electrophoresis. RT_PCR analysis used the following primers: *AtLIG6*, Actin_2f, 5′-TCCCTCAGCACATTCCAGCAGAT-3′, Actin_2r, 5′-AACGAATTCTGGACCTGCCTCATC-3′, rad51RTf, 5′-GTTCTTGAGAAGTCTTCAGAAGTTAG-3′, rad51rtr, 5′-GCTGAACCATCTACTTGCGCAACTAC-3′, and L6-2R, L6-2F, L6-4F and 699R, as used for genotyping.

Real-time RT-PCR analysis was performed on an iCycler thermocycler (Bio-Rad), as described previously (Dean *et al.*, 2009), using

iQ SYBR Green Supermix (Bio-Rad). Primer sets for ACTIN2 and AtRAD51 are as listed above. AtLIG6 primers were: LIG6_F1s, 5′-GCCGCTGACCATGAGAATATTGAGCT-3′; LIG6_R2, 5′-ATAGCTGAGCAACATCCCCAA-3′. AtLIG4 primers were: LIG4_F1s, 5′-CTTTAGTTTCGAAAAGCGAAATG-3′; LIG4_R2, 5′-CTTGTAGTGGATCCTCATGGA-3′.

Germination testing

Dry mature seed was harvested from soil-grown mutant and wild-type plants grown simultaneously. In all experiments seed from mutant and wild-type plants was produced in the same growth cycle. Seed was stored in sealed Eppendorf tubes in a controlled temperature facility at 15°C with a relative humidity of 15%. Seed was after-ripened for 2 months prior to germination testing. Immediately prior to germination testing, seeds were surface sterilized in 10% bleach (~0.5% sodium hypochlorite final concentration) and 1% Triton X100 for 5 min, followed by six washes with sterile water. Seeds used in ageing experiments were not surface sterilized because of the increased porosity of the seed coat. Seeds were sown on MS agar (M5519; Sigma-Aldrich, http://www.sigma-aldrich.com) supplemented with 1% sucrose and 0.8% plant agar (Duchefa, http://www.duchefa.com) in 90-mm Petri dishes, and germinated in a controlled environment room under a photoperiod of 16 h (light) at 22°C. Seeds were stratified for 2 days at 4°C in control experiments. Seeds were scored as germinated when the radicle had emerged from the seed coat. For low temperature germination experiments, Petri dishes of sown seeds were placed at 2°C in the light on MS plates for 14 days before transfer to standard growth conditions. Germination was followed at hourly or daily intervals, as appropriate, using a stereomicroscope to monitor radicle protrusion. Independent biological replicates were performed with different harvests of the wild-type and mutant lines for verification of results.

Seed ageing

Seed ageing was performed as previously described for Arabidopsis by Hay *et al.* (2003), at a temperature of 45°C with a relative moisture content of 10.8%. Several hundred (250 mg) seeds of each line were each placed in Eppendorf tubes with the lid removed and suspended over a saturated solution of KCl on an open platform in an air-tight box. The box was placed in a dark incubator at 45°C for between 0 and 8 days. After ageing, the seeds were either plated directly or stored in sealed Eppendorf tubes at 4°C following equilibration to the storage conditions described above. The relative moisture content was determined using a subset of aged seeds. The seeds were weighed before and after oven drying at 105°C for 18 h, after cooling over dry silica gel, to determine moisture loss. The relative moisture content was expressed as a percentage of the fresh weight.

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