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The *Arabidopsis AtLIG4* gene is required for the repair of DNA damage, but not for the integration of *Agrobacterium* T-DNA

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ABSTRACT

The joining of breaks in the chromosomal DNA backbone by ligases in processes of replication, recombination and repair plays a crucial role in the maintenance of genomic stability. Four ATP-dependent ligases, designated DNA ligases I–IV, have been identified in higher eukaryotes, and each one has distinct functions. In mammals and yeast, DNA ligase IV is exclusively involved in the repair of DNA double-strand breaks by non-homologous end joining. Recently, an *Arabidopsis thaliana* orthologue of the yeast and mammalian DNA ligase IV gene was found and termed *AtLIG4.* Here we describe the isolation and functional characterisation of a plant line with a T-DNA insertion in the *AtLIG4* gene. Plants homozygous for the T-DNA insertion did not display any growth or developmental defects and were fertile. However, mutant seedlings were hypersensitive to the DNA-damaging agents methyl methanesulfonate and X-rays, demonstrating that *AtLIG4* is required for the repair of DNA damage. Recently, we showed that a yeast *lig4* mutant is deficient in *Agrobacterium* T-DNA integration. However, using tumorigenesis and germline transformation assays, we found that the plant *AtLIG4* mutant is not impaired in T-DNA integration. Thus, in contrast to yeast, DNA ligase IV is not required for T-DNA integration in plants.

INTRODUCTION

The genomic integrity of cells is threatened by the formation of single- and double-stranded breaks in the chromosomal DNA. These may arise during replication, recombination or may be caused by DNA-damaging agents. DNA ligases play a critical role in the maintenance of genetic stability as they catalyse the joining of DNA molecules at sites of single- and double-stranded breaks by formation of new phosphodiester bonds in the DNA backbone. Prokaryotic cells express an NAD^+-driven ligase that catalyses the rejoining reactions during replication, recombination and DNA repair. Most eukaryotic organisms express DNA ligases that are driven by ATP. Moreover, they possess a variety of ligases each having a distinct funtion (1,2).

In yeast and mammalian cells, DNA ligase I is the main ligase involved in replication. It functions at the replication fork where it joins Okazaki fragments. However, DNA ligase I does not solely function in replication, as it is also required for base excision repair (3–5). An orthologue of the yeast and mammalian DNA ligase I gene has recently been cloned from the plant *Arabidopsis thaliana* and shown both to complement a mutation in the *Saccharomyces cerevisiae* DNA ligase I gene (*CDC9*) and to be most highly expressed in meristematic dividing tissue (6). These results suggested that, as in yeast and mammalian cells, this ligase plays an important role in DNA replication in plants.

Molecular cloning and biochemical analysis of cell extracts from different mammalian cell types revealed the presence of three other DNA ligases, termed DNA ligases II–IV. DNA ligase II has a lower molecular mass than DNA ligase III, shows a high degree of similarity in protein sequence to this ligase and may therefore be either a proteolysis product of the DNA ligase III protein or an alternative splicing product of the corresponding gene (7). An *in vivo* function for this ligase has yet to be discovered. The transcript of the DNA ligase III gene can be alternatively spliced, producing two products designated ligase IIIα and ligase IIIβ. Ligase IIIα is thought to be involved in base excision repair because it interacts with XRCC1, an essential protein of this repair pathway (8). Ligase IIIβ is only expressed in the testis and may function in meiotic recombination (9). A ligase has also been isolated from mammalian mitochondria. This ligase, which appears to function in the repair of damage to mitochondrial DNA, is most probably also a product of the ligase III gene, produced by the translation from an upstream start site, resulting in a

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protein with a potential mitochondrial targeting sequence (10). A detailed survey of the *S.cerevisiae* genome did not reveal the presence of a ligase III homologue.

Ligase IV was first detected in mammalian cell nuclei (11). The protein was found in a complex with the product of the *XRCC4* gene and the interaction required a region between the BRCT domains present in the C-terminus of ligase IV (12). Cells deficient for *XRCC4* are hypersensitive to ionising radiation and defective in V(D)J recombination, which is the process that assembles immunoglobulin and T-cell receptor genes in cells of the immune system (13,14). Targeted disruption of the ligase IV gene in mice leads to late embryonic lethality and is associated with defects in V(D)J recombination and apoptosis in the embryonic central nervous system (CNS) (15–17). These findings suggested that ligase IV is involved in the non-homologous end joining (NHEJ) pathway for repair of DNA double-strand breaks (DSBs). In higher eukaryotes, NHEJ is the pathway that is predominantly used for the repair of DSBs and the generation of immunoglobulins and T-cell receptor genes by V(D)J recombination. It is often associated with the loss of genetic information as it produces deletions and rearrangements. Besides the ligase IV–XRCC4 complex, this pathway involves the DNA–PK complex, consisting of the KU70–KU80 heterodimer and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and the multiprotein complex of RAD50–MRE11–NBS1 (18,19). Although in lower eukaryotes, such as yeast, homologous recombination is primarily used for DSB repair, the pathway for DSB repair by NHEJ is present. Homologues of the mammalian NHEJ genes, including ligase IV, have been found in yeast, indicating that NHEJ is highly conserved throughout eukaryotic evolution. Yeast cells lacking the ligase IV gene *LIG4* are viable, but are deficient in NHEJ (20–23). The product of the yeast *LIG4* gene interacts with ligase-interacting factor 1 (Lif1). Cells deficient for Lif1 also showed an increased sensitivity to ionising radiation, which is consistent with a role in DNA repair (24). Moreover, Lif1 was shown to stabilize DNA ligase IV and to recruit it to sites of DNA DSBs (25). Recently we found that DNA ligase IV also plays a role in the integration of *Agrobacterium* T-DNA in yeast.

Agrobacterium tumefaciens is a plant pathogen that induces crown gall disease on many dicotyledonous plant species at wound sites. The bacterium transfers an oncogenic segment of DNA, the transfer- or T-DNA, which is derived from a tumour-inducing (Ti)-plasmid in the bacterium, into plant cells (26). The T-DNA integrates at random positions into the plant genome by a process of non-homologous recombination (NHR) (27–29). *Agrobacterium* is used for the genetic engineering of plants, because the oncogenes in the T-DNA can be replaced by any other gene of interest without loss of transfer function. The host range of *Agrobacterium* is not restricted to plants. The bacterium is also able to transfer its T-DNA into cells of yeasts and filamentous fungi (30–32). In yeast, the T-DNA integrates by a mechanism of NHR similar to that in plants (33,34). Recently, we discovered that the NHEJ genes *RAD50*, *MRE11*, *XRS2*, *YKU70* and *LIG4* are involved in T-DNA integration in yeast (34). In plants, the histone H2A-1 gene *RAT5* was identified as being important for T-DNA integration (35), but whether the NHEJ genes play a role in T-DNA integration in plants has not yet been determined.

Recently, the sequence of the *A.thaliana* genome became available and a detailed analysis revealed the presence of genes with high homology to the mammalian and yeast ligase IV and XRCC4 genes, which were termed *AtLIG4* and *AtXRCC4*, respectively (36). The product of *AtLIG4*, a single-copy gene in the *Arabidopsis* genome, was shown to encode an ATP-dependent ligase, which interacted strongly with the AtXRCC4 protein via the BRCT domain in the C-terminus of the AtLIG4 protein. The expression of *AtLIG4* was induced by γ-radiation but not by UV-B irradiation, which implies a role for *AtLIG4* in DSB repair (36). Here we describe the isolation of an *A.thaliana* plant line in which the *AtLIG4* gene is disrupted by a T-DNA insertion. Plants homozygous for the T-DNA insertion are phenotypically indistinguishable from wild-type plants. In contrast to mammals where disruption of the gene encoding ligase IV is lethal (15,16), we obtained viable plants homozygous for the T-DNA insertion. These plants developed and grew normally, were fertile, but were hypersensitive to the DNA-damaging agent methyl methanesulfonate (MMS) and to X-rays, demonstrating that *AtLIG4* plays a role in the repair of DNA damage in *Arabidopsis*. In contrast to yeast, we found that *AtLIG4* is not required for integration of the *Agrobacterium* T-DNA into the plant genome.

MATERIALS AND METHODS

Isolation of the *Arabidopsis Atlig4* mutant

The collection of T-DNA insertion lines available at the University of Wisconsin *Arabidopsis* Knock-out Facility was screened using the T-DNA-specific primer JL-202 (37) and the *AtLIG4*-specific primers LG1 (5′-AGTTACTGGGAG-AGAAGACCGAGTCACTG-3′) and LG2 (5′-TTTCTTC-ACTAGAGGAGGAACTACGTCTG-3′) according to the guidelines available at http://www.biotech.wisc.edu/Arabidopsis/. A 2820 bp PCR product was generated using primers JL-202 and LG2 on the DNA pools. These primers were used in subsequent PCR rounds until individual plants harbouring a T-DNA insertion in *AtLIG4* were found. To map the insertion point of the T-DNA left end, the 2820 bp product was sequenced. The insertion point of the right T-DNA end was mapped after sequencing a 0.4 kb TAIL- (thermal asymmetric interlaced) PCR product generated on DNA from *AtLIG4*$^{-/-}$ plants using primers Tailp1 (5′-AAATGAGCACC-CAAAGAAGGCTC-3′), Tailp2 (5′-CTGTCAATTACCTCC-TTTTGCTC-3′) and Tailp3 (5′-CTTACAGTCTGAG-GTTTCCTCG-3′). The TAIL-PCR method was performed as described (38). For Southern blot analysis, DNA was extracted from individual plants using the DNeasy Mini Kit (Qiagen) and digested with XhoI. A 1216 bp fragment of the *AtLIG4* locus was amplified using primers LG18 (5′-GGTGCGACGCATGAGCAAG-3′) and LG19 (5′-ACT-GATGTATCGGATATCAAGGGC-3′), and used as a probe for Southern analysis. DNA and protein sequence analysis was performed using the Vector NTI Suite 6.0 program (Informax).

RT–PCR

Total RNA was extracted from young flowers and flower buds of wild-type (ecotype Wassilewskija) and *AtLIG4*$^{-/-}$ plants using the RNeasy kit (Qiagen) according to the supplied protocol. RT–PCR was performed as described (39) using 1 μg of total RNA for cDNA synthesis. The following primer pairs were used: p1 (5′-ATGACGGAGGAGATCAAATTCAGCG-3′) and p2 (5′-TGACCCACTTCATCTCCTGAGC-3′) to amplify a 496 bp fragment of the 5′ coding region of the *AtLIG4* cDNA; p3 (5′- GAAGAGGGTAATGCAGCTGG-3′) and p4 (5′-TCACCAGTCTTTGAGCAGTTCTCCG-3′) to amplify a 440 bp fragment of the 3′ coding region of the *AtLIG4* cDNA; 156m (5′-TCGCCTATAAATACGACG-GATCG-3′) and p6 (5′-TGCCCTTGATATCCGATACAT-CAG-3′) to amplify a 415 bp junction fragment of T-DNA and the 3′ coding region of the *AtLIG4* cDNA; and p5 (5′-GGGAACCTGGAGATCGTAGTGG-3′) and p6 to amplify the 5′ and 3′ coding regions of the *AtLIG4* cDNA that flank the T-DNA insertion. The primers RT-ROC5.1 (5′-CGGGAAG-GATCGTGATGGA-3′) and RT-ROC3.1 (CCAACCTTCTC-GATGGCCT-3′) were used to amplify a 400 bp fragment from the cyclophilin *AtROC5* cDNA. PCR conditions were as follows: 2 min at 94°C, 40 cycles of 20 s at 94°C, 30 s at 54°C and 45 s at 72°C, followed by 5 min at 72°C.

Assays for sensitivity to MMS and X-rays

Seed of wild-type (ecotype Wassilewskija) and *AtLIG4*$^{-/-}$ plants was sterilised as described (39) and germinated on 1/2 MS solid medium. Four days after germination, seedlings were transferred to liquid 1/2 MS medium containing 0, 0.006, 0.008 or 0.01% MMS (Sigma) and scored after 3 weeks. Sensitivity to X-rays was scored 3 weeks after irradiation of 4-day-old seedlings in 1/2 MS liquid medium. Seedlings were irradiated with 0, 80 or 150 Gy at 6 Gy/min using a 225 SMART X-ray machine as a source (Andrex SA, Copenhagen, Denmark) operated at 200 kV and 4 mA with a 1 mm aluminium filter.

Complementation analysis

A complementation construct was made by transferring an *AtLIG4* cDNA fragment to the binary vector pCAMBIA3301 (CAMBIA, Australia) to allow expression from a 35S cauliflower mosaic virus (CaMV) promoter. First, a 3254 bp NcoI–BglII 5′*AtLIG4*-containing fragment obtained from pCR2.1-TOPO-*AtLIG4* (36) was ligated into the unique NcoI and BglII sites of pCAMBIA3301. PCR was done on pCR2.1-TOPO-*AtLIG4* DNA using the Expand High Fidelity System (Boehringer Mannheim) and primers LG20 (5′-GAAGAGGGTAATGCAGCTGG-3′) and LG22StuI (5′-AAAAGGCCTTTTTCACCAGTCTTTGAGCAGTTCTCC-3′) to amplify the 3′ end of *AtLIG4*. A 450 bp PCR product was generated, cloned into pGEMT-Easy (Promega) and sequenced. A 482 bp BglII–HincII 3′ *AtLIG4*-containing fragment obtained from this clone was inserted into the BglII and NruI sites of pCAMBIA3301-5′*AtLIG4* to obtain pCAMBIA3301-*AtLIG4*. The binary vector, which carries a *bar* gene as a plant selectable marker, was introduced into *A.tumefaciens* strain LBA1115. *AtLIG4*$^{-/-}$ plants were transformed by the floral dip transformation method (40). T1 transformants were selected on 1/2 MS containing 20 mg/l phosphinothricine and 100 mg/l timentin. T2 plants were germinated on 1/2 MS containing 20 mg/l phosphinothricine and tested for sensitivity to MMS and X-rays as described above.

Tumorigenesis assay and floral dip transformation

The tumorigenesis or *in vitro* root transformation assay was performed according to the procedure described by Nam *et al.* (41). Floral dip transformation was performed as described by Clough and Bent (40), with the following modifications. Seed of wild-type and *AtLIG4*$^{-/-}$ plants was sterilised as described (39) and germinated on 1/2 MS solid medium. Twelve days after germination, seedlings were transferred to soil. Primary bolts were clipped and secondary bolts of ~ 5–10 cm in length were dipped in inoculation medium (5% sucrose, 0.02% Silwet L-77 and *Agrobacterium* cells resuspended to an OD600 of 0.8). The *Agrobacterium* strain LBA1115-(pSDM3010) was used for infection. The T-DNA from pSDM3010 carries a hygromycin resistance gene under the control of a nopaline synthase promoter and terminator. Plants were allowed to set seed and the number of seeds was determined according to the formula: weight (mg) × 50 = total number of seeds. Seed was plated on solid 1/2 MS medium containing 15 μg/ml hygromycin and 100 μg/ml timentin to kill off *Agrobacterium* cells. Hygromycin-resistant seedlings were scored 2 weeks after germination. In each experiment, 12 *AtLIG4*$^{-/-}$ and 12 wild-type plants were used for infection.

RESULTS

Isolation of an *Arabidopsis Atlig4* mutant

In order to identify plants containing a T-DNA insertion in *AtLIG4*, a collection of 60 480 T-DNA insertion lines was screened at the University of Wisconsin *Arabidopsis* Knock-out Facility as described (37). Eventually, we identified by PCR five individual plants harbouring a T-DNA insertion in the *AtLIG4* gene. Southern blot analysis with a segment of the *AtLIG4* gene as a probe revealed the presence of a 2.2 kb fragment in these plants that is characteristic for the *AtLIG4* gene with the T-DNA insertion. In three of the five plants, the 4.8 kb fragment of the wild-type *AtLIG4* gene was absent, indicating that these plants were homozygous for the T-DNA insertion (Fig. 1A and B). The other two plants were hemizygous for the insertion as they showed both the 2.2 and 4.8 kb fragment (data not shown). Southern blot analysis using an *nptII* probe indicated that the line contains three T-DNA copies in addition to the insertion in the *AtLIG4* gene (data not shown). The insertion point of the T-DNA left end was mapped by sequencing of the PCR product generated by primers JL-202 and LG2. The T-DNA left end turned out to be present in intron 17 of the *AtLIG4* gene. From the T-DNA left end, 45 bp were lost and 12 bp of filler DNA were present. No product could be amplified when primer p5 was used with a primer specific to the T-DNA right end. The T-DNA right end was therefore mapped by TAIL-PCR. The 0.4 kb TAIL-PCR product obtained was sequenced. This revealed that an intron 17 sequence was linked to 38 bp of filler DNA followed by a sequence identical to a part of the virulence region of a Ti-plasmid from *A.tumefaciens* strain C58, which was used for

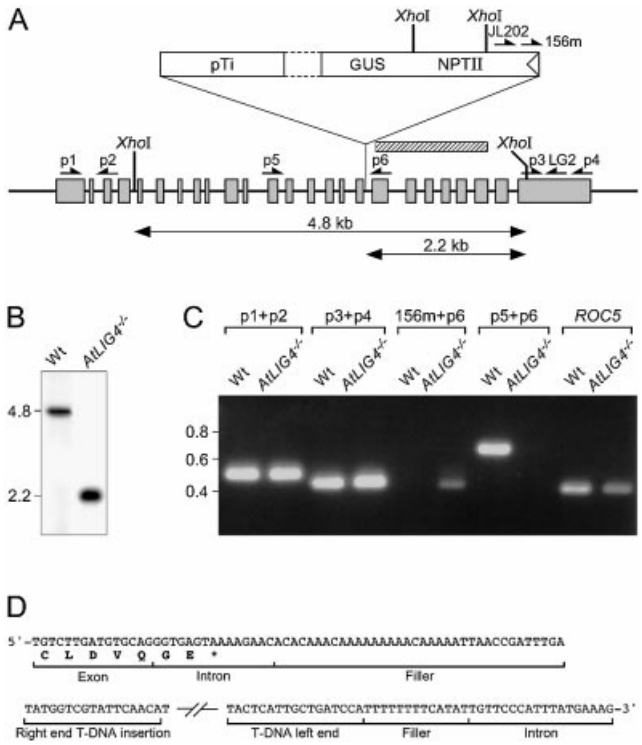

Figure 1. Molecular analysis of the T-DNA insertion in the *AtLIG4* locus. (**A**) Genomic organization of the *AtLIG4* locus. Exons (grey boxes) and the position of the T-DNA insertion in intron 17 of the *AtLIG4* gene are shown. Primers used for genotyping and RT–PCR analysis are indicated. (**B**) Southern analysis of the T-DNA insertion. The probe used for the blot (hatched box in A) was a 1216 bp fragment of the *AtLIG4* gene, which was generated by PCR. (**C**) RT–PCR analysis of the disrupted *AtLIG4* gene. RNA isolated from flower buds and young flowers of *AtLIG4*$^{-/-}$ and wild-type plants was used. Primers used in the RT–PCRs are indicated in (A). cDNA from the cyclophilin gene *AtROC5* was used as a positive control. (**D**) Sequence analysis of the T-DNA insertion. The nucleotide sequence of *AtLIG4* that flanks the right and left ends of the T-DNA insertion are shown. At the right end of the insertion, filler DNA and Ti-plasmid DNA fused to intron 17 of *AtLIG4* is presented. The nucleotide sequence at this site of the T-DNA insertion is aligned with the predicted amino acid sequence. At the left end of the T-DNA insertion, filler DNA and T-DNA sequence fused to intron 17 of *AtLIG4* are shown.

transformation. The T-DNA insertion event was accompanied by a 16 bp deletion of intron 17 DNA (Fig. 1A and D). RT–PCR analysis of the 5′ and 3′ *AtLIG4* coding regions that flank the T-DNA insertion revealed the presence of a transcript in both wild-type and *AtLIG4*$^{-/-}$ plants. However, RT–PCR analysis using primers flanking the insertion site detected a product in the wild-type, but not in *AtLIG4*$^{-/-}$ plants. Apparently, splicing at intron 17 does not occur in the *Atlig4* mutant and, consequently, the T-DNA is present in the *AtLIG* mRNA in the mutant (the PCR product in the mutant would be >10 kb in size and was therefore not detectable). This result was confirmed by RT–PCR analysis using a T-DNA-specific primer and a primer in the *3′ AtLIG4* coding region, with which a product was obtained in *AtLIG4*$^{-/-}$ plants, but not in wild-type plants (Fig. 1C). Sequence analysis showed that the transcript of the mutant *AtLIG4* gene could encode a truncated protein of 597 amino acids (the wild-type protein contains 1219 amino acids), of which the last two are derived from intron 17 (Fig. 1D). This putative protein will lack the nuclear localisation sequence and BRCT domains that are necessary for the interaction with AtXRCC4. If this protein is formed at all, it is most likely that it will be non-functional. Thus, the T-DNA insertion in the *AtLIG4* gene seemed to completely inactivate the gene (null phenotype).

In striking contrast to mice, where disruption of the ligase IV gene is embryonic lethal (15,16), we were able to obtain plants homozygous for the T-DNA insertion. These plants did not show any obvious defects in growth and development under normal growth conditions and were fertile. This suggests that the *AtLIG4* gene is not essential for plant growth, development and reproduction.

Atlig4 mutant plants are hypersensitive to MMS and X-rays

Yeast and mammalian cells deficient for ligase IV have defects in DSB repair and are sensitive to DNA-damaging agents. We tested whether disruption of the *AtLIG4* gene affects sensitivity of the plant to DNA-damaging agents by comparing growth of wild-type and *AtLIG4*$^{-/-}$ plants after exposure to MMS and X-rays. Seed was germinated on solid 1/2 MS medium and 4-day-old seedlings were transferred to liquid 1/2 MS medium supplemented with MMS and grown for 3 weeks. For X-ray sensitivity, seedlings were irradiated at the stated dosage in liquid medium and then grown further for 3 weeks. *AtLIG4*$^{-/-}$ plants were hypersensitive to MMS and X-rays (Fig. 2). In order to show that the sensitivity to MMS and X-rays correlates with the disruption of *AtLIG4*, we expressed the *AtLIG4* cDNA from the (CaMV) 35S promoter in *AtLIG4*$^{-/-}$ plants. Plants expressing the *AtLIG4* cDNA from the 35S promoter grew and developed more slowly on selection medium compared with wild-type and *AtLIG4*$^{-/-}$ plants that did not contain the 35S::*AtLIG4* construct (data not shown). However, the expression from the 35S promoter partially restored the X-ray sensitivity (data not shown), and completely restored the MMS sensitivity of *AtLIG4*$^{-/-}$ plants to levels similar to that of wild-type plants (Fig. 2A). These data showed that the hypersensitivity to MMS and X-rays is due to disruption of *AtLIG4* by the T-DNA insertion. Recently, similar phenotypes have been observed for plants deficient in *AtRAD50* (42), *AtMRE11* (43), *AtKU70* (44) and *AtKU80* (45), which all play a role in the repair of DNA damage. Our data show that, as in yeast and mammalian cells, *AtLIG4* also plays a role in the repair of DNA damage in plants.

AtLIG4 is not required for *Agrobacterium* T-DNA integration

Recently we found that a yeast *lig4* mutant is deficient in T-DNA integration by NHR, which showed that *LIG4* is required for T-DNA integration (34). To test whether the *AtLIG4* gene is similarly required for T-DNA integration in plants, we compared T-DNA integration in *AtLIG4*$^{-/-}$ plants with that in wild-type plants in two different *Agrobacterium* transformation assays. First, we performed an *in vitro* root segment tumorigenesis assay as described by Nam *et al.* (41). Sterile roots of 3-week-old wild-type and *AtLIG4*$^{-/-}$ plants grown in agar were cut into segments and inoculated with the virulent *Agrobacterium* strain A208. This strain harbours the nopaline-type Ti plasmid pTiT37 and induces the formation of large tumours on root segments. After 2 days co-cultivation,

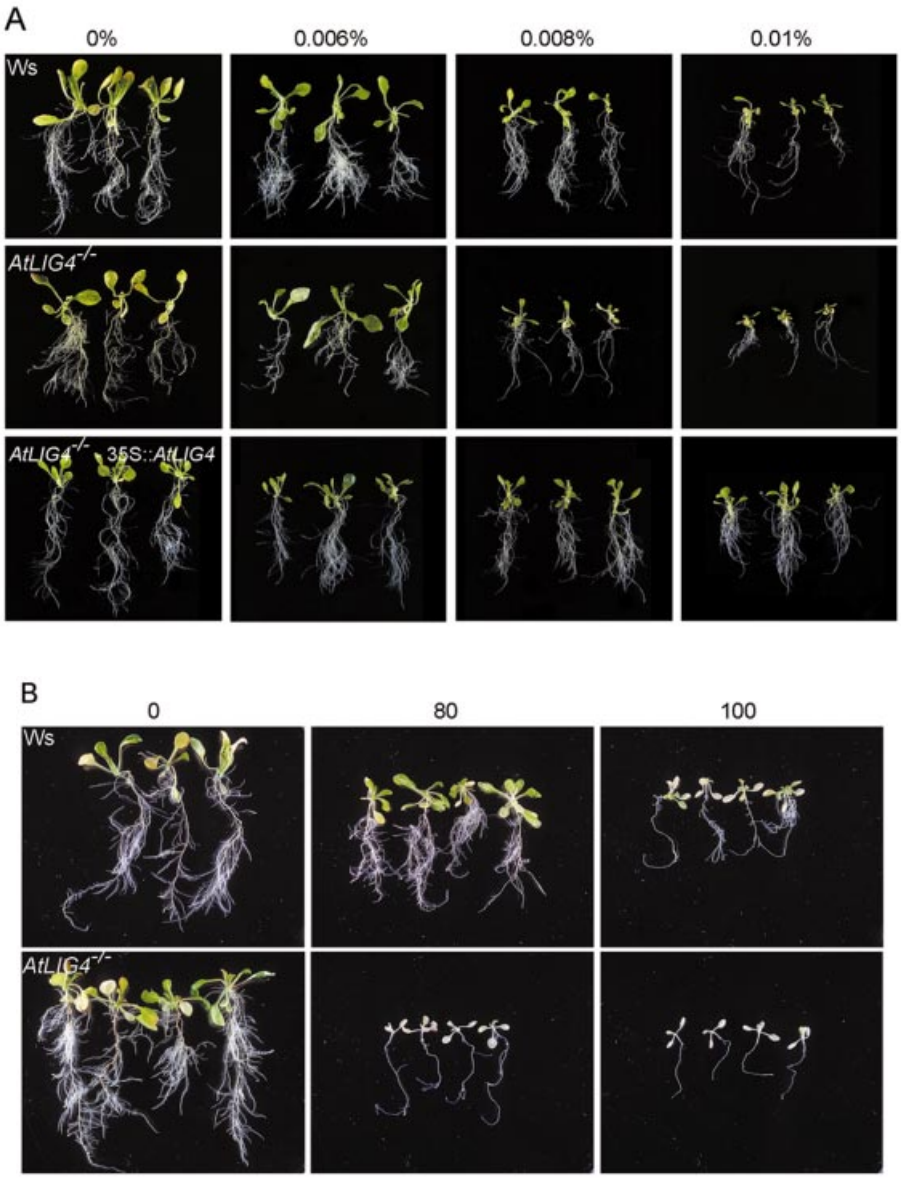

Figure 2. Hypersensitivity of *AtLIG4*$^{-/-}$ plants to DNA-damaging agents. (**A**) Response of wild-type, *AtLIG4*$^{-/-}$ and *AtLIG4*$^{-/-}$ –35S::*AtLIG4* plants to MMS. Four-day-old seedlings were transferred to medium containing different concentrations of MMS [stated in % (v/v) above the top row]. (**B**) Response of wild-type and *AtLIG4*$^{-/-}$ plants to X-rays. Four-day-old seedlings were transferred to liquid medium and irradiated with different doses (stated in Gy above the top row) of X-rays. For both experiments, phenotypes of plants were scored 3 weeks after treatment with the DNA-damaging agent.

an excess of *Agrobacterium* cells was washed away and the root segments were placed on solid culture medium lacking hormones. Tumorigenesis was scored as the percentage of infected root segments that produced any form of tumours. The efficiency of tumour formation of the *Atlig4* mutant did not differ significantly from that of the wild-type (Fig. 3A). Secondly, we performed a germline transformation using the floral dip method described by Clough and Bent (40). *AtLIG4*$^{-/-}$ and wild-type plants were transformed using the *Agrobacterium* strain LBA1115(pSDM3010) and plants resistant to hygromycin were selected as the T-DNA from the pSDM3010 binary vector carried a hygromycin resistance gene under control of the nopaline synthase promoter and terminator. The transformation efficiency was determined as the number of hygromycin-resistant seedlings per total number of plated seeds. The transformation efficiency of *AtLIG4*$^{-/-}$ plants did not differ significantly from that of the wild-type plants (Fig. 3B). Thus, in both the tumorigenesis and floral dip assay, *AtLIG4*$^{-/-}$ plants were as susceptible as wild-type plants to *Agrobacterium*-mediated transformation, which indicated that T-DNA integration is not blocked in *AtLIG4*$^{-/-}$ plants. Therefore, we conclude that *AtLIG4* is not required for efficient integration of *Agrobacterium* T-DNA.

Previously, we found in yeast that in the absence of ligase IV, the T-DNA, if integrated at all, preferably formed repeat structures at unknown chomosomal loci. Although the absence of ligase IV in plants did not affect the efficiency of T-DNA integration, it may alter the features of T-DNA integration. To

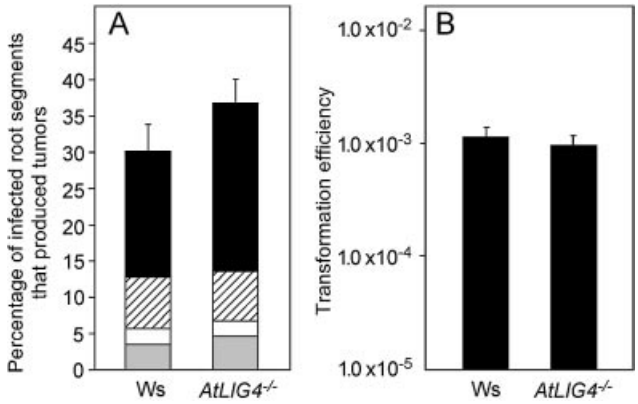

Figure 3. *Agrobacterium*-mediated transformation of *AtLIG4*$^{-/-}$ and wild-type plants. (**A**) Tumorigenesis assay. Sterile root segments of 3-week-old *AtLIG4*$^{-/-}$ and wild-type plants were infected with the virulent *Agrobacterium* strain A208. Two days after infection, root segments were washed and tranferred to solid culture medium lacking phytohormones. Tumour formation was scored 4 weeks after infection and represented as the percentage of root segments that produced tumours. Tumours were morphologically scored as large green (black), small green (hatched), large yellow (white) or small yellow (grey). (**B**) Floral dip assay. Two-week-old *AtLIG4*$^{-/-}$ and wild-type seedlings were transferred to soil. Primary bolts were clipped and secondary bolts of 5–10 cm in length were dipped into inoculation medium containing *Agrobacterium* strain LBA1115-(pSDM3010). Seed from infected plants was harvested, weighed to determine the total number of seeds (1 mg ~ 50 seeds) and plated on 1/2 MS medium containing hygromycin. The number of hygromycin-resistant seedlings was scored 2 weeks after germination. The transformation efficiency is represented as the number of hygromycin-resistant seedlings per total number of seeds. In each experiment, 12 *AtLIG4*$^{-/-}$ plants and 12 wild-type plants were tested.

investigate this, we subjected 12 transgenic wild-type and 12 *AtLIG4*$^{-/-}$ plant lines, which were generated by floral dip transformation using *Agrobacterium* LBA1115(pSDM3010), to Southern blot analysis. The number of T-DNA copies and T-DNA repeat structures found in the genome of wild-type and *AtLIG4*$^{-/-}$ plants was comparable (Table 1 and data not shown). We then used TAIL-PCR to isolate the T-DNA left end–plant DNA junction sites for a number of T-DNA insertions in wild-type and *AtLIG4*$^{-/-}$ plants. Sequences obtained from the TAIL-PCR products were analysed and used in BLAST searches against the *Arabidopsis* genome. We were able to determine the position of eight T-DNA insertions in wild-type and *AtLIG4*$^{-/-}$ transformants, respectively (Table 1 and data not shown). In both the wild-type and *AtLIG4*$^{-/-}$ plants, T-DNA insertions were found at different positions on all chromosomes, but preferentially at intergenic regions as described before (46). No insertions were found at regions of repeated DNA (rDNA, telomeres and retro-elements). Moreover, in both the wild-type and *AtLIG4*$^{-/-}$ plants, truncation of the T-DNA left end (0–27 and 0–23 bp), microhomology between the T-DNA left end and the insertion site (0–8 and 0–4 bp) and formation of filler DNA (0–9 and 0–112 bp) were seen after integration, as described before for T-DNA integration in plants (Table 1) (27–29). Taken together, these data indicated that the features of T-DNA integration in wild-type and *AtLIG4*$^{-/-}$ plants were comparable. Thus, the absence of *AtLIG4* also did not affect the features of T-DNA integration.

Table 1. Features of T-DNA integration in wild-type and *AtLIG4*$^{-/-}$ plants

T-DNA insertion	Wild type	*AtLIG*$^{-/-}$
T-DNA copies[a]	38 (12)	39 (12)
T-DNA repeats[b]	11 (38)	12 (39)
Coding region[c]	1 (8)	2 (8)
Intergenic region[c]	7 (8)	6 (8)
Repeated DNA[c]	0 (8)	0 (8)
Deletion at LB[d]	0–27 bp (8)	0–23 bp (8)
Microhomology at LB[d]	0–8 bp (8)	0–4 bp (8)
Filler DNA[d]	0–9 bp (8)	0–112 bp (8)

[a]The number of T-DNA copies per total number of analysed plants (numbers in parentheses).
[b]The total number of T-DNA repeats (direct and inverted repeats) per total number of T-DNA insertions (number in parentheses).
[c]The number of T-DNA insertions at specific regions per total number of analysed T-DNA insertions (number in parentheses).
[d]Deletions, microhomology and filler DNA at the T-DNA left border (LB) in bp per total number of T-DNA insertions (number in parentheses).

DISCUSSION

In this study, we describe the isolation and characterization of an *A.thaliana* plant line in which the gene encoding ligase IV, *AtLIG4*, is disrupted by a T-DNA insertion. We were able to obtain plants homozygous for the T-DNA insertion. These plants were viable, although they lacked the wild-type *AtLIG4* mRNA. A transcript containing the T-DNA insertion was detected by RT–PCR, which may encode a truncated version of the AtLIG4 protein. However, it is unlikely that this truncated protein would be functional as it would lack the nuclear localization sequence and the BRCT domain. Consequently, it would not be imported into the nucleus and could not interact with the XRCC4 protein, which is essential for ligase IV activity in NHEJ in yeast and mammals (12,24,47). Therefore, we predict that the disruption line will have a null phenotype. We were not able to detect any significant differences in phenotype between *AtLIG4*$^{-/-}$ and wild-type plants. *AtLIG4*$^{-/-}$ plants germinated, developed and grew normally and were fertile. However, *AtLIG4*$^{-/-}$ plants were more sensitive to treatments with the DNA-damaging agents MMS and X-rays. Thus, as in yeast and mammalian cells, *AtLIG4* plays a role in the repair of DNA damage.

Targeted disruption of the ligase IV gene in mice resulted in late embryonic lethality (15,16). However, we demonstrate here that, as in yeast (20–23), inactivation of the ligase IV gene is not lethal in plants. This may be explained by differences in function between the ligase IV proteins from plants and yeast on one side and mammals on the other side. Mammalian, yeast and plants cells that lack ligase IV are all deficient in the repair of DNA damage. However, in mice, deficiency for ligase IV additionally leads to defects in V(D)J recombination, and thus a compromised immune response, and cell death by apoptosis in the CNS (16,17). These two additional developmental effects may explain why the absence of ligase IV leads to embryonic lethality in mammals. However, as plants lack both an immune and nervous system, these consequences of a ligase IV deficiency are absent in plants. Indeed, the presence of ligase IV is not required for development of the *Arabidopsis* embryo.

We have tested the role of the *AtLIG4* gene in DSB repair in *Arabidopsis* by comparing the response of *AtLIG4*$^{-/-}$ and

wild-type plants to treatment with the DNA-damaging agents X-rays and MMS. We found that *AtLIG4*$^{-/-}$ seedlings are hypersensitive to both X-rays and MMS. The sensitivity to MMS of *Atlig4*$^{-/-}$ seedlings seemed to be comparable with that of the previoulsy identified *Atmre11-2* (43) and *Atku70* (44) mutants as we did not observe any differences in experiments where we compared the sensitivity to MMS of *Atlig4*$^{-/-}$, *Atku70*$^{-/-}$ and *Atmre11–2*$^{-/-}$ seedlings (data not shown). *AtLIG4*$^{-/-}$ seedlings seemed to be more sensitive to X-rays than MMS. After exposure to 0.006% MMS and 80 Gy of X-rays, wild-type seedlings were still able to grow and develop. However, at these dosages, growth and development of *AtLIG4*$^{-/-}$ seedlings were more affected by treatment with X-rays than MMS (Fig. 2). X-rays induce predominantly DSBs, whereas treatment with MMS leads to abasic lesions and single-strand breaks, which may be converted to DSBs during DNA replication in S phase. In yeast, DNA ligase IV has been shown to play an exclusive role in the repair of DSBs by NHEJ (20–23). The finding that *AtLIG4*$^{-/-}$ seedlings are more sensitive to X-rays than MMS is therefore consistent with a role for *AtLIG4* in the repair of DSBs by NHEJ in plants.

Components of the NHEJ machinery in yeast, mammalian and plant cells have been shown to control telomere length. For example, yeast and mammalian cells lacking Ku70 have shortened telomeres (48–50), whereas plants deficient for *KU70* have longer telomeres when compared with the corresponding wild-type plants (44,51). However, neither in yeast nor in mammalian cells did mutations in *LIG4* affect telomere length (20,50). We analysed telomere length in *AtLIG4*$^{-/-}$ plants as described previously (52) and did not observe any significant difference in telomere length between these plants and wild-type plants (data not shown). This indicated that, as in yeast and mammals, DNA ligase IV does not play a role in telomere length regulation.

Recently we found that a yeast *lig4* mutant is deficient in T-DNA integration by NHR, which showed that *LIG4* is required for T-DNA integration. In the *lig4* mutant, the T-DNA, if integrated at all, preferably formed repeat structures at unknown chomosomal loci (34). In contrast to our findings in yeast, we demonstrated here that in plants *AtLIG4* is not required for T-DNA integration. Neither the efficiency nor the features of T-DNA integration, meaning the number of T-DNA copies, T-DNA repeat structures and distribution of T-DNAs in the plant genome, were affected by the absence of ligase IV in plants. How may these differences between yeast and plants in the requirement for DNA ligase IV in T-DNA integration be explained?

Could the function of *Arabidopsis* DNA ligase IV be taken over by another ligase? *AtLIG4* is a single-copy gene in the *Arabidopsis* genome and is not a member of a gene family (36). Homologues of the mammalian DNA ligase II and III have not been found yet in plants and therefore their role in T-DNA integration remains unclear. West *et al.* (36) reported the identification of a novel putative DNA ligase in *Oryza sativa* and *Arabidopsis.* Further characterisation of this DNA ligase may show whether it is a homologue of the mammalian DNA ligase II or III or a novel plant-specific DNA ligase and whether it plays a role in T-DNA integration. The best characterized DNA ligase in plants so far is DNA ligase I. We may therefore only speculate about a possible role for DNA ligase I in T-DNA integration in plants.

Functional analysis of the *Arabidopsis* DNA ligase I gene showed that it complements a mutation in the yeast ligase I gene *CDC9*, implicating a function in replication (6). Biochemical analysis of the *Arabidopsis* and *Saccharomyces* DNA ligase I demonstrated that not only nicks in DNA, but also double-stranded DNA molecules with blunt and cohesive ends are good substrates for ligation (53,54). Joining of blunt and cohesive ends is normally required for the repair of DSBs by NHEJ, a process that exclusively involves DNA ligase IV in yeast and mammalian cells. Indeed, the yeast *CDC9* was unable to complement the deficiency of a *lig4* mutant in a plasmid DSB rejoining assay, demonstrating that *in vivo* DNA ligase I does not play a major role in DSB repair in yeast (21). To our knowledge, these types of experiments have not yet been done with the *Arabidopsis* DNA ligase I and so it is theoretically possible that the plant DNA ligase I can join DNA DSBs. By using cell cycle phase-specific inhibitors, Villemont *et al.* (55) showed that T-DNA integration predominantly occurs during the S phase in plants. This suggests that DNA replication may be a prerequisite for T-DNA integration and that the T-DNA may become integrated during the repair of DNA damage formed during DNA replication. The *Arabidopsis* DNA ligase I has been implicated to function in DNA replication (6). Morover, in plants, expression of the DNA ligase I gene was shown to be highest in flowers, which primarily consist of meristematic cells undergoing mitosis and meiosis. This is consistent with a role for the plant DNA ligase I in DNA replication and a possible function in T-DNA integration during S phase. Consequently, we cannot rule out that *Arabidopsis* DNA ligase I may be able to take over the role of DNA ligase IV in T-DNA integration.

We have found previously that besides Lig4, other NHEJ proteins such as Yku70, Rad50 and Mre11 are required for T-DNA integration by NHR in yeast. Orthologues of the yeast *KU70*, *RAD50* and *MRE11* genes have been found in plants, and plant lines in which these genes are disrupted by a T-DNA insertion are viable and became available (43,44,51,52). In view of our finding that *AtLIG4* is not required for T-DNA integration in plants, a crucial next step would be to investigate whether mutations in these other NHEJ genes, as in yeast, do affect T-DNA integration. This should further increase our understanding of the mechanism of T-DNA integration in plants.

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