Chapter 2

Characterization of *Arabidopsis thaliana* NHEJ mutants

Qi Jia, Paul Bundock, B. Sylvia de Pater and Paul J.J. Hooykaas
Abstract

Eukaryotic organisms use two distinct pathways to repair DNA double-strand breaks (DSBs), namely non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ rather than HR is the major pathway for DSB repair in somatic cells of higher eukaryotes, and it may also be the major route whereby T-DNA integrates into the genome during Agrobacterium-mediated transformation. The NHEJ pathway has been well characterized in yeast and mammals. In order to study NHEJ in Arabidopsis thaliana, three plants lines with a T-DNA insertion in AtKu80, AtKu70 or AtLig4 genes were functionally characterized. Western blot analysis demonstrated that knockout of either AtKu80 or AtKu70 resulted in loss of the other protein, showing that heterodimer formation is needed for stability of the proteins. Plants homozygous for the T-DNA insertion were phenotypically indistinguishable from wild-type plants and were fertile. However, these mutants were hypersensitive to the genotoxic agent bleomycin, resulting in more DSBs as quantified in comet assays. They had lower end joining efficiency, suggesting that NHEJ is a critical pathway for DSB repair in plants. Both Atku mutants and a previously isolated Atmre11 mutant were impaired in Agrobacterium T-DNA integration via floral dip transformation, whereas the Atlig4 mutant was hardly affected, indicating that AtKu80, AtKu70 and AtMre11 play an important role in T-DNA integration in Arabidopsis, but that another ligase may substitute for AtLig4. The frequency of gene targeting was not increased in the Atku80, Atku70 and Atlig4 mutants, but it was increased at least 10 fold in the Atmre11 mutant compared with the wild-type.

Introduction

The genomic integrity of organisms is threatened by exogenous genotoxic agents, such as reactive oxygen species, ionizing radiation and chemicals, as well as by endogenous cellular processes, such as transcription and replication (1-3). The most threatening damage is the occurrence of a double-strand break (DSB) in the chromosomal DNA. To maintain genetic stability, organisms have developed two main independent pathways for DNA repair. One is the homologous recombination (HR) pathway involving extensive DNA sequence homology between the interacting molecules, and the other is the non-homologous end joining (NHEJ) pathway acting independently of significant homology. HR occurs during the late S to G2 phases of the cell cycle when the sister chromatid is in close proximity as repair template. On the other hand, NHEJ does not require a homologous chromosome and can function throughout the cell cycle (4). Generally, HR is mainly used in bacteria and lower eukaryotes, whereas NHEJ is used predominantly in higher eukaryotes (5;6). NHEJ is more error-prone than HR and often produces short deletions and insertions, whereas HR may lead to large-scale genetic rearrangements with repetitive sequence elements in large genomes. Emerging evidence suggests that the relative balance of the two pathways is tuned
to minimize the mutagenesis as a consequence of repair (7).

In response to DNA damage, the Mre11-Rad50-Xrs2 (MRX) complex in yeast and its counterpart in mammals, called Mre11-Rad50-Nbs1 (MRN), function early as a key player in the DNA damage sensing, signaling and repair mechanism of both HR and NHEJ pathways (8;9). The pathway of NHEJ in S. cerevisiae and mammals has been extensively characterized. In mammals, conserved proteins that are involved in NHEJ include the following: DNA-dependent protein kinase (DNA-PK), Ku70, Ku80, DNA ligase IV (Lig4), Xrcc4 and Cernunnos/XLF (10;11). Orthologs of these proteins have been identified also in yeast, fungi and plants with the exception of DNA-PKcs, which does not seem to play a role in NHEJ in these organisms. Ku is an abundant non-histone nuclear protein in human cells. However Ku is also found in the membrane and cytoplasm (12;13). Ku can shuttle from the cytoplasm to the nucleus dependent on the cell cycle status and external stimuli, like irradiation, alkylating agents and hormones such as somatostatin (14;15). Ku has an extremely high affinity to DNA ends, and thus rapidly binds to DSBs in living cells (4). DSB repair is initiated by the recognition and binding of the Ku heterodimer consisting of Ku70 and Ku80 to the exposed DNA ends. In mammals the Ku heterodimer recruits DNA-PKcs and activates its kinase activity (16-18). Once Ku is bound to the DNA ends, it can improve the binding equilibrium of the nuclease (Artemis·DNA-PKcs), the polymerases (μ and λ) and the ligase complex (XLF-XRCC4·Lig4) (19;20). In this way, Ku serves as a scaffold of the subsequent protein assembly and stabilizes their enzymatic activities at a DNA end (11). The following step is that the Lig4/XRCC4/XLF complex catalyzes the ligation and seals the joint (21), thereby restoring the genomic integrity. Lig4 has an exclusive function in NHEJ by forming a complex with XRCC4 through the BRCT domain in the C-terminus of Lig4 (22). This complex associates with Pol χ family polymerases, Pol μ, Pol λ and terminal transferase, which fill in the short gaps generated during DNA end alignment and processing (23;24). The Lig4/XRCC4 complex also has an impact on the association of Cernunnos/ XLF which promotes the ligation of mismatched and noncohesive DNA ends (21;25). The NHEJ pathway is Ku-dependent and is also called classical NHEJ (C-NHEJ). Another NHEJ pathway has been identified in C-NHEJ deficient cells, which is Ku-independent, called back-up NHEJ (B-NHEJ) (26).

Integration of transgenes in lower eukaryotes depends on NHEJ and HR DNA repair pathways (27;28). Mutations in the NHEJ pathway favour the HR pathway and thereby increase the frequency of gene targeting, which is a useful technique using HR to change an endogenous gene (27-31). Gene targeting is very rare in higher eukaryotes, like animals and plants. Estimates of GT frequencies in several different plant species vary from $10^{-4}$ to $10^{-6}$ (32-40). Therefore, it would be an advantage if also in plants higher gene targeting frequencies could be achieved via inactivation of NHEJ.

Previous studies have reported on the identification of Arabidopsis NHEJ mutants (9;41-46). Most of these mutants were in the ecotype Wassilewskija (Ws). Those mutants display hypersensitivity to agents that induce DSBs, indicating the requirement of those
genes under genotoxic stress. Here we characterized three Columbia-0 (Col-0) Arabidopsis lines containing a T-DNA insertion in the \textit{AtKu70}, \textit{AtKu80} and \textit{AtLig4} genes. The genome of the Col-0 ecotype has been sequenced completely and T-DNA insertion lines are available in most genes, including those involved in DNA repair, so that in the future we can analyze other DNA repair mutants including combinations of DNA repair pathways. Plants homozygous for the T-DNA insertions were analyzed for sensitivity to DNA damaging agents and it was shown directly that these proteins function in end joining in plants. \textit{Agrobacterium}-mediated transformation and the frequency of gene targeting was analyzed in these NHEJ mutants and the \textit{Atku70} and \textit{Atmre11} mutants (ecotype Ws) previously described (9;41).

\textbf{Material and methods}

\textbf{Characterization of the Arabidopsis T-DNA insertion mutants}
\textit{Atku80}, \textit{Atku70} and \textit{Atlig4} T-DNA insertion lines were obtained from the SALK T-DNA collection (SALK\_016627, SALK\_123114, SALK\_044027, respectively). Information about them is available at http://signal.salk.edu/cgi-bin/tdnaexpress (47). The T-DNA insertion site was mapped with a T-DNA Left Border (LB) specific primer LBA1 and a gene-specific primer. Pairs of gene-specific primers around the insertion site were used to determine whether the plants were homozygous or heterozygous for the T-DNA insertion, and the PCR products were sequenced. T-DNA right border (RB) specific primers (Sp205, Sp206) were used to detect the T-DNA Right Border/ vector junction. The sequences of all the primers are listed in Table 1. For Southern blot analysis, DNA was extracted from individual plants using the CTAB DNA isolation protocol (48) and digested with HindIII (\textit{Atku70} and \textit{Atlig4}) or PstI (\textit{Atku80}). DNA (5\textmu g) was ran on a 0.7\% agarose gel and transferred onto positively charged Hybond-N membrane (Amersham Biosciences). The hybridization and detection procedures were done according to the DIG protocol from Roche Applied Sciences. The DIG probe was produced using the PCR DIG Labeling Mix (Roche) with specific primers SP271 and SP272 that amplified an 850-bp fragment from the T-DNA of pROK2. Characterization of the \textit{Atku70} and \textit{Atmre11} mutants (ecotype Ws) was described previously (9;41).

\textbf{Quantitative reverse-transcription PCR (Q-RT-PCR)}
Leaves of 2-week-old wild-type (ecotype Col-0), \textit{Atku80}, \textit{Atku70} and \textit{Atlig4} plants were ground under liquid N\textsubscript{2} in a Tissue-Lyser (Retch). Total RNA was extracted from the leaf powder using the RNeasy kit (Qiagen) according to the supplied protocol. Residual DNA was removed from the RNA samples with DNaseI (Ambion) in the presence of RNase inhibitor (Promega). RNA was quantified and 1 \textmu g of RNA was used to make cDNA templates using iScript cDNA synthesis kit according to the manufacturer’s instructions (Bio-Rad). Quantitative real-time PCR (Q-PCR) analyses were done using the iQ\textsuperscript{™} SYBR\textsuperscript{®} Green
Supermix (Bio-Rad). Specific fragments (about 200 bp) were amplified by pairs of primers around the T-DNA insertion sites using a DNA Engine Thermal Cycler (MJ Research) equipped with a Chromo4 real-time PCR detection system (Bio-Rad). The sequences of the primers are listed in Table 1. The cycling parameters were 95°C for 3 min, 40 cycles of (95°C for 1 min, 60°C for 40s), 72°C for 10 min. All sample values were normalized to the values of the housekeeping gene Roc1 (Primers Roc5.2, Roc3.3) and were presented as relative expression ratios. The value of the wild-type was set on 1.

Table 1. Sequences of primers used for characterization of T-DNA insertion lines and Q-PCR.

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<tr>
<th>Name</th>
<th>Locus</th>
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<tr>
<td>LBa1</td>
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<td>AtKu80</td>
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<td>AtLig4</td>
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<tr>
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<td>5'-CGGCGGTCTGACCATCGT-3'</td>
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Western blotting

Leaves from plants grown in soil for 3 weeks were ground under liquid N$_2$ in a Tissue-Lyser (Retch). One hundred µl protein extraction buffer (50 mM Tris-HCl pH 7.5; 2 mM EDTA; 0.2 mM PMSF; 1 mM DTT; 1×Protease inhibitor cocktail Complete®, EDTA free) was added to 50 mg of tissue powder. Soluble protein was isolated by centrifugation at 4°C. The protein concentration was determined using the BIO-RAD protein assay reagent. Approximately 10 µg soluble proteins were electrophoresed on 10% SDS/PAGE (49). The fractioned products were semi-dry blotted onto BA85 nitrocellulose membrane (Whatman). Equal loading of the gels and quality of protein preparations were checked by staining extra sets of gels with Coomassie Brilliant Blue R250 (not shown). Blots were blocked for 3 hr in 5% nonfat dry milk in PBST (10 mM NaPO$_4$, pH 7.4; 120 mM NaCl; 2.7 mM KCl; 0.05% v/v Tween 20) and incubated overnight at 4°C with peptide antibodies (1:1000) in the same buffer. Rabbit anti-Ku80 and anti-Ku70 polyclonal antibodies were raised against synthetic peptides corresponding to small domains of the proteins that exhibit sequence diversity and were affinity chromatography purified (Eurogentec). The sequences of the synthetic peptides for AtKu80 and AtKu70 were NH2-LLRDKPSGSDDEDN-+C-CONH$_2$ and NH2-ELDPDDVFRDEDEDP-+C-CONH$_2$, respectively, with a C-terminal coupling on the added cystein. The blots were washed 4 times with PBST and incubated for 3 h with anti-rabbit HRP antibodies (1:7500) (Promega). The blots were washed 4 times with PBST and detection was performed using LumiGLO™ chemoluminescence detection kit (Cell Signalling).

Assays for sensitivity to bleomycin and methyl methane sulphonate (MMS)

Seeds from Col-0 wild-type and the Atku80, Atku70 and Atlig4 mutants were surface-sterilized as described (50) and were germinated on solidified ½ MS medium (invented by Murashige and Skoog, containing MS salts with macro- and micronutrients, vitamins, FeNaEDTA, myo-inostol, MES, sucrose, Daishin Agar) (51). For bleomycin and MMS sensitivity, 4-day-old seedlings were transferred to liquid ½ MS medium or liquid ½ MS medium containing 0.2 µg/ml and 0.4 µg/ml Bleocin™ antibiotic (Calbiochem), or 0.007% and 0.01% (v/v) MMS (Sigma). The seedlings were scored after 2 weeks of growth at 21 °C in a growth chamber (16 h light/8 h dark, 2500 lux at 70% humidity). After this period, fresh weight (compared with controls) was determined by weighing the seedlings in batches of 20 in triplicate.

Comet assay

DSBs were detected by a neutral comet assay as described previously (52) with minor modifications. Plant nuclei were embedded in 1% low melting point Ultrapure™ agarose-1000 (Invitrogen) to make a mini gel on microscopic slides according to the protocol. Nuclei were subjected to lysis in high salt (2.5 M NaCl, 10 mM Tris-HCl, pH7.5, 100 mM EDTA) for 20 min at room temperature (N/N protocol). Equilibration for 3×5
min in 1×TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.4) on ice was followed by electrophoresis at 4 °C in TBE buffer for 15 min at 30 V (1 V/cm), 15–17 mA. Dry agarose gels were stained with 15 ul ethidium bromide (5 μg/ml) and immediately evaluated with a Zeiss Axioplan 2 imaging fluorescence microscope (Zeiss, Germany) using the DsRed channel (excitation at 510 nm, emission at 595 nm). Images of comets were captured at a 40-fold magnification by an AxioCam MRC5 digital camera (Zeiss, Germany). The comet analysis was carried out by comet scoring software CometScore™ (Tritek Corporation). The percentage of tail DNA (%tail-DNA) was used as a measure of DNA damage. The %tail-DNA was measured at 3 time points: 0 h, 2 h, 24 h for 1 μg/ml bleomycin treatment and in the seedlings which had 24 h recovery in ½ MS after 24 h treatment. Measurements were performed for 4 independent gel replicas and approximately 100 comets analyzed for each treatment.

Isolation of Arabidopsis mesophyll protoplasts

Arabidopsis Col-0 was either grown in a greenhouse at 21°C (16 h photoperiod) or in a culture chamber (21°C, 50% relative humidity, 16 h photoperiod). Rosette leaves (~1 g) from plants that were 3 to 5 weeks old were collected, rinsed with deionized water and briefly dried. The leaves were cut into 0.5 to 1 mm strips with a razor blade, placed into a sterile Petri dish containing 15 ml of filter-sterilized enzyme solution [1.5% (w/v) cellulose R10, 0.4% (w/v) macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7, 10 mM \( \text{CaCl}_2 \), 0.1% (w/v) BSA] and incubated 2-3 h in the dark at 28°C. Then the protoplasts were filtered through a 50 μm mesh to remove the undigested material and transferred to a round-bottom Falcon tube. The solution was centrifuged for 5 min at 600 rpm to pellet the protoplasts. The supernatant which contained broken cells was discarded. The protoplasts were gently washed twice by 15 ml cold W5 solution (154 mM NaCl, 125 mM \( \text{CaCl}_2 \), 5 mM KCl, 2 mM MES pH 5.7), and resuspended in cold W5 solution to a final concentration of 2×10^5 cells/ml and kept on ice for 30 min. Just before starting transfection, protoplasts were collected from the W5 solution by centrifugation and were resuspended to a density of 2×10^5 cells/ml in MMg solution (0.4 M mannitol, 15 mM MgCl$_2$, 4 mM MES pH 5.7) at room temperature.

![Figure 1. Schematic diagram of pART7-HA-GFP. The primers for Q-PCR are shown by arrows.](image)
**End joining assay**

Plasmid pART7-HA-GFP(S65T) was linearized by cleavage with BamHI (Figure 1). Fresh protoplasts prepared from leaves were transformed with either linear or circular plasmid DNA by the polyethylene glycol (PEG) transformation protocol (53). In each experiment, 2×10⁴ protoplasts were transformed with 2 µg of plasmid. Recircularization of the linear plasmid in protoplasts by the NHEJ pathway was analyzed. DNA was extracted from protoplasts (at 0 h and 20 h) and was used to quantify rejoining by Q-PCR. The DNA extraction protocol and the cycling parameters of Q-PCR were the same as mentioned above. Two pairs of primers were used: one pair (q8+q9) was flanking the enzyme digestion site (BamHI); the other pair (q10+q11) was localized in GFP (Figure 1). When the plasmid is circular, both of them give products. When the plasmid is cleaved by BamHI, the first pair of primers will not give a product, whereas the second will still give products. The efficiency of end joining is presented by the ratio of PCR products using q8+q9 primers and q10+11 primers in comparison with the controls. The value of the wild-type was set on 1. Q-PCR was performed as three replicates and the assays were performed in triplicate.

**Root transformation**

Root transformation was performed as described by Nam et al. (54). Sterile roots of 3-week-old wild-type and mutants grown in agar were cut into segments and inoculated with *Agrobacterium* strain ATΔvirD2 with wild-type VirD2 on a plasmid harboring a β-glucuronidase (GUS)-expressing binary vector (55). After 2 days co-cultivation, an excess of *Agrobacterium* cells was washed away. Some of the root segments were stained with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) to measure the transient expression. GUS activity was determined as the number of blue spots per seedling. The residual root segments were plated on callus-induction medium containing kanamycin for 3-4 weeks to test callus formation as measure for T-DNA integration (45). Stable transformation was scored as infected root segments that produced any form of callus.

**Floral dip transformation**

Floral dip transformation was performed according to the procedure described by Clough and Bent (56). The *Agrobacterium* strain AGL1 (pSDM3834) (34) was used for infection. Plasmid pSDM3834 is a pCambia 1200 derivative (hpt selection marker). Seeds were harvested from the dry plants after maturation and plated on solid MA medium (57) without sucrose containing 15 µg/ml hygromycin, 100 µg/ml timentin (to kill *Agrobacterium* cells) and 100 mg/ml nystatin (to prevent growth of fungi). Hygromycin-resistant seedlings were scored 2 weeks after germination and transformation frequency was determined (50 seeds weigh approximately 1mg) (43).

**Gene targeting**

In order to test the frequency of gene targeting in the *Atku70* mutant (ecotype Ws) (41) and
the *Atmre11* mutant (ecotype Ws) (9) which were previously characterized by our lab, floral dip transformation was performed with *Agrobacterium* strain AGL1 (pSDM1502) (original name of the plasmid is pSDM) for gene targeting using the protoporphyrinogen oxidase (*PPO*) system (32). For the three NHEJ mutants (ecotype Col-0), floral dip transformation was performed with *Agrobacterium* strain AGL1 (pSDM3900). Plasmid pSDM3900 is a pCambia 3200 derivative (phosphinothricin (ppt) selection marker). The *PPO* sequences used for gene targeting were cloned from the Col-0 ecotype and mutations were introduced to obtain resistance to the herbicide butafenacil. About 1 gram seeds were plated on solid MA medium without sucrose containing 15 µg/ml ppt, 100 µg/ml timentin and 100 µg/ml nystatin to determine the transformation frequency. The rest of the seeds were all sowed on solid MA medium without sucrose containing 50 µM butafenacil, 100 µg/ml timentin and 100 µg/ml nystatin to identify gene targeting events. The butafenacil-resistant plants were analyzed with Southern blot analysis to determine if they represented true gene targeting (TGT) events or contained extra T-DNA integration (Figure 2).

**Figure 2.** The design for the targeted modification of the Arabidopsis *PPO* locus. The white box marked *PPO* represents the *PPO* coding region, and the thick black lines represent flanking plant genomic DNA. The thin lines indicate the T-DNA sequences. The two mutations conferring Butafenacil resistance are indicated as stars. The *BAR* resistance gene is linked to the truncated 5′ΔPPO of the T-DNA (LB for left border and RB for right border). The probes and the restriction enzyme digestion sites used for Southern blot analysis are also shown.

**Results**

**Isolation and characterization of the *Atku80 Atku70* and *Atlig4* mutants**

In order to study the role of AtKu80, AtKu70 and AtLig4 in *Agrobacterium* transformation and gene targeting, *Arabidopsis thaliana* (ecotype Col-0) homozygous T-DNA insertion
mutants were obtained from the Salk collection and characterized (Figure 3). When two gene-specific primers flanking the insertion site were used, PCR products were amplified for the wild-type and heterozygotes. No PCR products were obtained for homozygous mutants by using two gene-specific primers, because the PCR products in the mutant would be >10 kb in size and would not be detectable. When T-DNA-specific primer LBa1 was used in combination with one gene-specific primer, PCR products for the T-DNA-insertion mutants were amplified, whereas no PCR products were obtained for the wild-type. We identified

**Figure 3.** Molecular analysis of the NHEJ T-DNA insertion lines.
Genomic organization of the *AtKu80* (A), *AtKu70* (B) and *AtLig4* (C) locus. Inserted T-DNAs are indicated. Exons are shown as black boxes. 3' and 5' UTRs are shown as gray boxes. Introns are shown as lines. The primers used for genotyping and Q-RT-PCR analysis are indicated. The probe (▬) and the restriction enzyme digestion sites (P for PstI, H for HindIII) used for Southern blot analysis are also indicated. Genomic DNA sequences (gDNA) flanking the T-DNA insertion are shown in italic. pROK2 denotes the vector part of this binary vector.
homozygous mutants harboring a T-DNA insertion for each of the three genes (data not shown) in the next generation. The insertion point of the T-DNA left border (LB) was mapped by sequencing of the PCR products generated by LBa1 in combination with one of the gene-specific primers. For Atlig4, PCR products were produced by LBa1 combined with both gene-specific primers (Sp268, Sp269), indicating the insertion of a T-DNA inverted repeat with the LB at both ends. For Atku80 and Atku70, no PCR products were obtained using LBa1 or the RB primer in combination with the other gene-specific primer, but PCR products (600bp) were amplified by using primers spanning the RB/vector junction (Sp205 and Sp206). That indicated that the RB was integrated with extra vector pBin-ROK DNA. Therefore, the RB integration site could not be mapped in the Atku80 and Atku70 mutants.

![Southern blot analysis of T-DNA insertion mutants.](image)

The genomic DNA was digested by PstI (Atku80) or HindIII (Atku70 and Atlig4). An 850-bp amplified region of the T-DNA of pROK2, was used as probe. M: λHindIII Marker. 1, 2, 3 and 4 indicate individual progeny plants of Atku70 and Atlig4.

The genomic DNA was digested by the PstI (Atku80) or HindIII (Atku70 and Atlig4) for Southern blotting (Figure 4). If there is one T-DNA inserted in the correct locus, bands with the following sizes will be detected on the blot: Atku80: 32 kb; Atku70: 3.2 kb; Atlig4: 2.1 kb and 3.8 kb. If T-DNAs are also present in other loci, additional bands probably with different sizes will be detected. If there are 2 or more T-DNAs inserted in one locus as direct repeat, an additional band of 4317 bp, representing a complete T-DNA, will be detected. If there are 2 or more T-DNAs inserted in one locus as indirect repeat, an additional band of 3634 bp will be detected. Sequencing results and Southern blot analysis using a pROK2 probe indicated that the T-DNAs were all inserted at the position as reported by the Salk database. A detailed characterization of the T-DNA insertions is shown in Figure 3. The Atku80 line contained 1 T-DNA insertion. The LB end of the T-DNA in Atku80 was integrated into intron 10, had lost 7 base pairs (bps) and had incorporated 43 bps filler DNA during integration. The Atku70 line contained more than 2 T-DNA copies. The LB end of the T-DNA in Atku70 was integrated into exon 8 and had 28 bps filler DNA.
Atlig4 line contained 2 T-DNA copies inserted as an inverted repeat. The two LB ends of the T-DNAs in Atlig4 were integrated into exon 6, one missing 18 bps and the other missing 11 bps. The Atlig4 gene itself had lost 25 bps. There were extra T-DNAs present in other loci in plants 2 and 3 of the Atlig4 line, but these were absent in plants 1 and 4, which were used for further analysis (Figure 4).

We did not observe any dwarf or sterile phenotype in these Arabidopsis mutant lines. Also no obvious differences in growth, flowering or senescence were observed between mutants and wild-type plants. A somewhat delayed germination was the only phenotypic change exhibited by the Atku80 mutant. This is in accordance with former reports about the phenotype of mutants with T-DNA insertions in these genes in the ecotype Wassilewskija (Ws) (41;43;46).

Expression analysis

In order to check expression of the mutated genes, Q-RT-PCR analysis was performed for each T-DNA insertion line using primers flanking the insertion site. This resulted in a product for each gene in the wild-type, but not in the corresponding T-DNA insertion mutant (Figure 5). This indicated that in the insertion mutants no stable mRNA is produced from the mutated gene and confirms that the plants are indeed homozygous mutants. Compared with the wild-type, each mutant had a higher expression of the two other intact NHEJ genes. Thus, there could be some form of feedback regulation for the expression of these three genes.

![Figure 5](image_url)

**Figure 5.** RNA expression of the NHEJ genes AtKu80, AtKu70 and AtLig4, determined by Q-RT-PCR, in wild-type and mutant plants. All the sample values were normalized to Rob values and the ratios were obtained in triplicate. The values of the wild-type were set on 1. (□ wild-type; ■ Atku80; — Atku70; □ Atlig4)

Western blot analysis was performed on extracts from wild-type, Atku80, Atku70 and Atlig4 plants using polyclonal peptide antibodies raised against AtKu80 or AtKu70. An 80-kD band, the predicted size of the AtKu80, was present in the wild-type and Atlig4 plants, but not detectable in the Atku80 mutant with anti-Ku80 antibodies. A very weak signal was found in the Atku70 mutant. A 70-kD band, the predicted size of the AtKu70 protein, was present in the wild-type and Atlig4 plants, but not detectable in the Atku70 mutant with
anti-Ku70 antibodies and the signal was very weak in the *Atku80* mutant (Figure 6). Loss of one of the Ku subunits thus resulted in a significant decrease in the amounts of the other subunit according to the western blot data. This indicated that heterodimerization of the Ku proteins is required for Ku protein stabilization in plants, as was shown before in mammals (14;58).

![Western blot analysis](image)

**Figure 6.** Western blot analysis of leaf protein samples from the *Atku80*, *Atku70*, *Atlig4* mutants and wild-type (Col-0) plants using anti-AtKu70 antibody (A) and anti-AtKu80 antibody (B). Detected Ku70 and Ku80 proteins are indicated with arrows. Around 10 µg of soluble leaf protein was loaded for each plant line.

**Sensitivity to genotoxic agents**

Mammalian cell lines, yeast strains and Arabidopsis mutants lacking Ku80, Ku70 or Lig4 have defects in DSB repair and are sensitive to DNA-damaging agents (10;41;43;59). We tested whether disruption of these genes in the Col-0 background also affects sensitivity of the plants to DNA-damaging agents by comparing growth of the wild-type and these NHEJ mutants during exposure to bleomycin or MMS. Bleomycin is a radiomimetic chemical that induces DNA double strand breaks (DSBs) (60). MMS is a monofunctional alkylating agent that induces N-alkyl lesions and DNA single strand breaks (SSBs) that can be converted into DSBs during replication (61). As expected, the *Atku80*, *Atku70* and *Atlig4* mutants turned out to be hypersensitive to bleomycin and the *Atku70* and *Atlig4* mutants seemed also somewhat more sensitive to MMS. To quantify this, the fresh weight of seedlings was determined after 2 weeks of continuous treatment. Compared with wild-type seedlings, NHEJ mutated seedlings showed growth retardation in the presence of these two genotoxic agents (Figure 7). In the presence of bleomycin, the fresh weight of *Atku80* was 2-fold less than that of the wild-type and the fresh weight of *Atku70* and *Atlig4* was about 4-fold less than that of the wild-type. The fresh weight of all seedlings was lower after growth in the presence of MMS compared to bleomycin. According to the student’s T-test, the *Atku70* and *Atlig4* mutants had a significantly lower weight than the wild-type grown in the presence of MMS, but there was no significant difference between the wild-type and the *Atku80* mutant. These data corroborated that AtKu80, AtKu70 and AtLig4 are main components
of the DSB repair machinery and that the \textit{Atku70} and \textit{Atlig4} mutants are more sensitive to genotoxic agents than the \textit{Atku80} mutant.

In order to quantify the DNA damage in these mutants, comet assays were performed. For each treatment, around 100 randomly chosen nuclei were analyzed by using CometScore™.

**Figure 7.** Hypersensitivity of \textit{Atku80} \textit{Atku70} and \textit{Atlig4} plants to DNA-damaging treatments.

(A) Phenotypes of wild-type (Col-0) plants and \textit{Atku80}, \textit{Atku70} and \textit{Atlig4} mutants to bleomycin or MMS treatment. Four-day-old seedlings were transferred to liquid \(\frac{1}{2}\) MS medium (control) or \(\frac{1}{2}\) MS medium containing different concentrations of bleomycin (Bleo) or MMS and were scored 2 weeks after germination.

(B) Fresh weight of 2-week-old wild-type plants and \textit{Atku80}, \textit{Atku70} and \textit{Atlig4} mutants treated with 0.2 µg/ml bleomycin or 0.007% MMS. For each treatment 20 seedlings were weighed in triplicate. Fresh weight of the plants grown for 2 weeks without bleomycin or MMS was set at 1. Student's test: * P<0.05, ** P<0.001 (comparing mutants with the wild type of the same treatment).

(\textbullet\textcolor{black}{\textbullet} wild-type; \textbullet\textcolor{red}{\textbullet} \textit{Atku80}; \textbullet\textcolor{green}{\textbullet} \textit{Atku70}; \textbullet\textcolor{blue}{\textbullet} \textit{Atlig4})
The percentage DNA in the tail is related to the amount of DNA damage. Scoring was done by dividing the nuclei into three groups according to percentage DNA in the tail (<5, 5~10, >10). Thereafter, for each treatment the proportion of the nuclei in each group was determined. The results showed that the genomic DNA of the *Atku80, Atku70* and *Atlig4* mutants was more damaged than that of the wild-type, especially after treatment with bleomycin for 24h (Figure 8). Already, after 2h treatment, more DNA damage was found in the nuclei of the *Atku80* and *Atku70* mutants, indicating that the genome of the *Atku80* and *Atku70* mutant was damaged quicker and thus that repair was slower than in the *Atlig4* mutant and the wild-type (Figure 8). Interestingly, the NHEJ mutants had less DNA damage after 24h recovery compared to the situation before recovery, though they still had more DNA damage than the wild-type, especially the *Atku70* and *Atku80* mutants (Figure 8). This showed that a slow back-up DNA repair pathway must exist for repair of DNA damage in the NHEJ mutant plants.

![Figure 8. Comet assay.](image)

(A) The fraction of DNA in comet tails (%tail-DNA) was used as a measure of DNA damage in wild-type (Col-0), *Atku80, Atku70* and *Atlig4* plants. For each treatment, around 100 nuclei were analyzed. The means of %tail-DNA after bleomycin treatment are shown. 

(B) According to the value of %tail-DNA, nuclei were divided into three groups (<5, 5~10, >10). The proportions of these groups are shown for wild type (Col-0), *Atku80, Atku70* and *Atlig4* plants after: no treatment; b2h: 2h bleomycin treatment; b24h: 24h bleomycin treatment; br: 24h bleomycin treatment followed by 24h recovery.
**End joining activity**

To directly test the function of \textit{AtKu80}, \textit{AtKu70} and \textit{AtLig4} in end joining, we used an \textit{in vivo} plasmid rejoining assay to quantify the capacity of the protoplasts to repair restriction enzyme generated DSBs. To this end, we transformed protoplasts from leaves with circular (control) or BamHI linearised plasmid DNA. BamHI digests the plasmid DNA in the N-terminal part of the GFP coding sequence. Rejoining of linear plasmid \textit{in vivo} will result in GFP expression. GFP fluorescence was indeed detected in the wild-type protoplasts which were transformed with linearized plasmid. But it was difficult to quantify the difference of GFP expression between the wild-type and the mutants under the fluorescence microscope. Therefore, we analyzed the rejoining efficiency by Q-PCR, using primers around the BamHI site compared to primers in the GFP coding region. The results showed that the rejoining efficiencies were reduced by half in the \textit{Atku80}, \textit{Atku70} and \textit{Atlig4} mutants compared with the wild-type (Figure 9). It proved directly that \textit{AtKu80}, \textit{AtKu70} and \textit{AtLig4} are involved in NHEJ.

![Figure 9. Plasmid end-joining assay.](image)

Rejoined plasmid DNA with respect to total plasmid DNA in the wild-type Col-0 was set on 1. Values of end joining in the mutants are given relative to that of the wild-type.

**T-DNA integration**

Double strand break repair forms an important mechanism for the integration of \textit{Agrobacterium} T-DNA in the genome. In yeast non-homologous T-DNA is integrated by NHEJ (27), but the results obtained in plants so far are variable (43;45;59;62). To test whether the \textit{AtKu70}, \textit{AtKu80} and \textit{AtLig4} genes are required for T-DNA integration, we compared T-DNA integration in those mutants and in wild-type plants using two different \textit{Agrobacterium} transformation assays.

First, \textit{in vitro} root transformation was performed as described by Nam \textit{et al} (54), using a binary vector with both a \textit{GUS} gene and the \textit{nptII} gene (for Kanamycin resistance). The transient GUS activity was determined as the number of blue spots per root segment. T-DNA integration was scored as the percentage of infected root segments that produced any form of callus on selective callus-inducing medium. In principle, for each plant line, the transient GUS activity, which reflects expression of non-integrated T-DNA, should be at the same
level, whereas callus formation will be reduced in mutants affected in T-DNA integration. Our results showed no significant difference, neither in transient GUS expression nor in callus formation between the NHEJ mutants and the wild-type (data not shown).

Secondly, we transformed germline cells using the floral dip method as described by Clough and Bent (56). The transformation frequency here was determined as the number of hygromycin resistant seedlings per total number of plated seeds. In this assay, the transformation frequency of \textit{Atku80} and \textit{Atku70} plants turned out to be reduced significantly to 20 percent of the wild-type transformation frequency, whereas the transformation frequency of \textit{Atlig4} plants was not significantly reduced (Figure 10). It indicated that AtKu80 and AtKu70, but not AtLig4, are required for efficient \textit{Agrobacterium} T-DNA integration in plant germline cells.

**Gene targeting**

T-DNA can not only be integrated by non-homologous recombination, but also at low frequency by homologous recombination (gene targeting). The number of gene targeting (GT) events was shown to be increased after disruption of NHEJ in lower eukaryotes, such as yeast and fungi (28;29;63;64). In order to test whether disruption of NHEJ also increased gene targeting frequency in plants NHEJ mutants were used in \textit{Agrobacterium} floral dip transformation using a T-DNA with an incomplete mutated \textit{PPO} gene with homology to the plant genome (Figure 2). If the targeted endogenous \textit{PPO} gene is replaced by the mutated \textit{PPO} of the T-DNA, the plants become resistant to butafenacil. If there are extra T-DNAs randomly inserted in other loci of the genome, the plants will also become resistant to phosphinotricin (ppt). Besides the Col-0 mutants characterized here, previously described
Atku70 and Atmre11 mutants were used of the ecotype Ws (9;41), because no Atmre11 mutants could be obtained for the Col-0 ecotype.

**Table 2. Transformation and gene targeting frequencies.**

<table>
<thead>
<tr>
<th>Plant lines</th>
<th>Transformants tested</th>
<th>Butafenacil resistant</th>
<th>ppt resistant</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Col-0)</td>
<td>2600</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Atku80 (Col-0)</td>
<td>1143</td>
<td>0</td>
<td>-</td>
<td>0.23</td>
</tr>
<tr>
<td>Atku70 (Col-0)</td>
<td>1086</td>
<td>0</td>
<td>-</td>
<td>0.24</td>
</tr>
<tr>
<td>Atlig4 (Col-0)</td>
<td>1537</td>
<td>0</td>
<td>-</td>
<td>0.66</td>
</tr>
<tr>
<td>WT (Ws)</td>
<td>10927</td>
<td>0</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Atku70 (Ws)</td>
<td>980</td>
<td>0</td>
<td>-</td>
<td>0.23</td>
</tr>
<tr>
<td>Atmre11 (Ws)</td>
<td>3625</td>
<td>3</td>
<td>3</td>
<td>0.21</td>
</tr>
</tbody>
</table>

The numbers of different events found in gene targeting experiments. The transformation frequency was shown as the ratios compared with the wild-type and the value of the wild-type was set on 1.

Firstly, 1 g of seeds was used to determine the transformation frequency by plating on ppt media (Table 2). In all 5 mutants the frequency was lower compared to the wild-type as already described above for the three Col-0 mutants (Figure 10). The remaining of the seeds was plated with selection for butafenacil resistance. The theoretical number of transformants in these seed batches was calculated from the transformation frequency.

There were 2 butafenacil-resistant plants found in 2600 transformants of the wild-type of ecotype Col-0. PCR analysis showed that both plants represent true gene targeting (TGT) events (data not shown). One of these plants is also ppt resistant, indicating the presence of extra T-DNA copies. There were no GT events found in around 1000 transformants of the Atku70, Atku80 and Atlig4 Col-0 mutants (Table 2), indicating that the gene targeting frequency was not significantly increased in those mutants compared with the wild-type.

Subsequently, the Ws wild-type and mutants were used for a GT experiment. In about $10^4$ wild-type transformants no GT event was observed. Since the transformation frequency of Atku70 (ecotype Ws) was rather low only about 1000 transformants were obtained and no GT events were observed. However, there were 3 butafenacil-resistant plants found in 3625 transformants of the Atmre11 mutant (ecotype Ws). Therefore, the frequency of gene targeting was increased at least 10 fold in the Atmre11 mutant compared with that in the Ws wild-type. Initial PCR analysis showed that GT had occurred in these plants (results not shown). These plants were further analyzed by Southern blotting.

The genomic DNA of pools of progeny plants of the 3 plant lines was digested by KpnI and Ncol for Southern blotting (Figure 11). With the 5’ PPO probe and the 3’ PPO probe, a band of 11.6 kb was detected for the wild-type allele in all lanes. In most progeny plants of all three lines a band of 2 kb representing the gene targeting allele was detected with the 5’
PPO probe. With the 3’ PPO probe, a band of 9.6 kb representing the gene targeting allele was only detected in line IV. The results showed that the plant lines III and V of Atmre11 represented ectopic gene targeting (EGT) events, whereas plant IV represented a true gene targeting (TGT) event. All of the three plant lines had extra bands with the 3’, the 5’ and the BAR probe, and moreover, they were ppt-resistant, suggesting that they all contained extra T-DNA insertions. In principle, individual homozygous plants for the modified copy of the PPO locus would be segregated from the progenies in case of TGT. But it was strange that only the bands for T-DNA and 5’ PPO segregated, and the bands for the wild-type PPO did not segregate.

**Figure11.** Gene targeting analysis of butafenacil resistant Atmre11 lines. Southern blot analysis was performed for progeny of the three individual plant lines (III, IV and V) of the Atmre11 mutant. P is for positive control (PPO resistant plant) and W is for the wild-type. The probe used for the top panel is probe 5’ PPO, the probe for the middle panel is probe 3’ PPO and probe for the bottom panel is probe Bar. The sizes for the bands of the marker from the top to the bottom are as follows: 23130 kb, 9416 kb, 6557 kb, 4361 kb, 2322 kb, and 2027 kb.

**Discussion**

Here, we isolated and characterized three *Arabidopsis thaliana* NHEJ mutants in which AtKu80, AtKu70 and AtLig4 were inactivated through a T-DNA insertion. The mouse is the only other multicellular organism in which the effects of inactivation of the Ku80, Ku70 and Lig4 genes have been studied in detail. Mice lacking Ku80 or Ku70 are fertile, but they show growth retardation and have immuno-deficiencies due to defects in V(D)J recombination.
Mice lacking Lig4 show late embryonic lethality associated with extensive apoptosis in the embryonic central nervous system (65-68). Yeasts and filamentous fungi with mutations in the NHEJ genes have no obvious growth phenotype (28;30;31;64;69). The Arabidopsis thaliana mutants homozygous for those mutations, which we obtained, showed no obvious growth phenotypes or sterility. Only the AtKu80 mutant showed a phenotype in which it differed from the wild-type, i.e. delayed germination. However, the NHEJ mutants all did show a clear hypersensitivity to the DNA damaging agent bleomycin. Thus, as in yeast and mammalian cells, AtKu80, AtKu70 and AtLig4 play a role in the repair of DNA damage, especially in DSBs repair. In mice, the Ku80, Ku70 and Lig4 genes are likewise involved in DNA damage repair; but here they may have additional functions that are necessary for normal development and may be involved in cell growth, DNA replication, G1-S transition and Bax mediated apoptosis (70-73). Lig4 was also found to play a role in apoptosis in mammalian cells (74). In plants and lower eukaryotes, Ku80, Ku70 and Lig4 genes may lack these additional functions and therefore show no phenotype under standard growth conditions. Three Atmre11 mutants (ecotype Col-0) were also ordered by us from the Salk T-DNA collection (Atmre11c-1: SALK_028450, Atmre11c-2: SALK_0554418, Atmre11c-3: SALK_067823). No homozygous mutant was obtained for the Atmre11c-2 mutant. The homozygous Atmre11c-1 and Atmre11c-3 mutants were obtained. They were dwarf and sterile (data not shown), similar to the phenotype of the Atmre11-1 mutant (ecotype Ws) described by Bundock et al. (9). Since no seeds were obtained from these Atmre11 mutants (ecotype Col-0), no further analysis was done with these mutants. Therefore the Atmre11 (ecotype Ws) mutant that was previously isolated was used for gene targeting experiments.

We have tested the role of AtKu80, AtKu70 and AtLig4 genes in DNA repair in Arabidopsis thaliana (ecotype Col-0) by comparing the response of the T-DNA insertion mutants and the wild-type to treatment with the DNA-damaging agents, bleomycin and MMS. The NHEJ mutants turned out to be hypersensitive to bleomycin. The Atku70 and Atlig4 mutants are also sensitive to MMS. Similar phenotypes have been observed for Arabidopsis (ecotype Ws) deficient in AtKu80 (46), AtKu70 (41) and AtLig4 (43). Atku80, Atku70 and Atlig4 seedlings seem to be more sensitive to bleomycin than to MMS compared to wild-type plants. Bleomycin is a radiomimetic drug that will induce predominantly DSBs, whereas MMS is a methylating agent that will cause abasic sites and SSBs, that may be converted to DSBs during DNA replication in S phase (75). This indicated that AtKu80, AtKu70 and AtLig4 genes may play a more important role in DSBs repair than SSBs repair in plants. Previously, it was found that a Atlig4 mutant is less sensitive to MMS than to X-rays, which also induces mainly DSBs (43). These results confirmed that Ku80, Ku70 and Lig4 all play a role in the repair of DSBs by NHEJ in plants, as they do in yeast and mammalian cells.

It has been reported that Ku80 and Ku70 need to be heterodimerized as a ring to bind DNA ends and repair DNA damage (11;76). We showed that dimerization is necessary for stabilization of the heterodimer. Therefore, the Atku80 and Atku70 mutants are expected to have similar phenotypes. However, Atku70 plants seem to be more sensitive to bleomycin
than *Atku80* plants (Figure 6). It was also reported that there are some differences in the phenotype of *Ku70* - and *Ku80*-knockout mice (77). One recent report showed that deleting Ku70 had milder effects than deleting Ku80 in p53-mutant mice. The authors suggested that Ku80 may function outside the Ku heterodimer to suppress cancer caused by translocation in mice (78). In plants, Ku70 and Ku80 may also have additional functions besides their role as Ku heterodimer in NHEJ. Since the T-DNA is inserted in the very end of the *AtKu80* gene in the *Atku80* mutant, it is possible that a small amount of truncated Ku80 remaining in the *Atku80* mutant heterodimerized with Ku70 to promote DNA end joining.

We investigated the RNA expression of *AtKu80* in the *Atku80* mutant by RT-PCR using primers located in the N-terminal part of the coding region before the T-DNA insertion site. The results showed that there are products detectable (data not shown). Possibly a truncated protein, which still may retain partial function, is produced at low level causing the *Atku80* mutant to stand stress better than the *Atku70* mutant. Western blot was performed using the antibody that in principle could detect a C-terminal truncated Ku80 protein. But no Ku80-related bands with a smaller MW were detectable on the western blot, indicating that no truncated protein was produced or that the amount was too low for detection. Alternatively, the presence of the complete or partial mRNAs may have a regulatory role in the plant cell.

Double strand break repair mechanisms are hypothesized to control the integration of *Agrobacterium* T-DNA in plants. In yeast non-homologous T-DNA is integrated by NHEJ (27), but the results obtained in plants so far are variable (43;45;59;62). In order to test whether *AtKu80*, *AtKu70* and *AtLig4* are involved in T-DNA integration, we performed root transformation assays and floral dip transformation and calculated the transformation frequency. The results of floral dip transformation showed that the transformation frequency is highly reduced in the *Atku70* and *Atku80* mutants. However, the results of root transformation showed that the NHEJ mutants have similar transformation frequency compared to the wild-type. Thus T-DNA integration occurs equally well in root cells of the wild-type and the NHEJ mutants. Li et al. (2005) also performed root transformation assays and they found that Ku80-deficient mutants have fewer tumors than wild-type plants (45). It could be that calli induced on the wound site of roots are derived from multiple cells and that high transformation frequencies mask differences between wild-type plants and mutants in our experiments. Previously, our group has shown the essential role of NHEJ proteins including the Ku70/Ku80 complex and Lig4 in T-DNA integration in the yeast *S.cerevisiae* (27;28). The AtLig4 gene was not required for efficient integration of *Agrobacterium* T-DNA (ecotype Ws) (43). Recently, Windhofer et al. (2007) showed that low levels of DNA Ligase III and IV are sufficient for effective NHEJ in mammal cells (79). Plants may have similar mechanisms. When AtLig4 is deficient, another DNA ligase of plants (such as DNA ligase I or VI) may take over the function of AtLig4 in T-DNA integration.

Although the efficiencies of T-DNA integration in the *Atku70* and *Atku80* mutants are highly reduced, random T-DNA integration still occurs in these mutants. In addition, the frequency of gene targeting was not increased in these NHEJ mutants. In yeast and fungi,
the frequency of gene targeting was increased dramatically in absence of NHEJ components, such as Ku70/Ku80, Lig4, Mre11, Rad50, Xrs2 (28;29;31;63;64). It could be that C-NHEJ is the only NHEJ pathway in lower eukaryotes, whereas there must be one or several back-up NHEJ (B-NHEJ) pathways in plants. When C-NHEJ is blocked, the back-up pathways are still robust to take the function of DNA repair. Deficiency of AtMre11 resulted in an increase in gene targeting, suggesting the MRN complex could function at the initial step of end joining and be involved not only in C-NHEJ, but also in other pathways. We will explore B-NHEJ further in the future.

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