Role of non-homologous end-joining in T-DNA integration in Arabidopsis thaliana

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Chapter 2

Agrobacterium T-DNA integration in *Arabidopsis* non-homologous end-joining mutants

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Abstract

Arabidopsis thaliana parp3 or *xrcc1* mutant was isolated, and subsequently double and triple mutants (*parp1parp3*, *ku80xrcc1*, *parp1parp2parp3*) were obtained by crossing. Treatments with DNA damaging agents showed that *PARP3* and *XRCC1* are involved in DNA repair. We further examined transient and stable root transformation frequencies of these mutants after co-cultivation with *Agrobacterium*. Knocking out components of either the c-NHEJ or b-NHEJ pathway, did not lead to a significant decrease in root transformation. However, the *ku80xrcc1* and *ku80p1p2* mutants, in which both c-NHEJ and b-NHEJ pathways are inactivated, showed a significant decrease in stable root transformation efficiency. However, no significant differences were observed in transient transformation. These results indicate that T-DNA integration requires the known NHEJ repair pathways for optimal transformation, but that there must still be other important factors and/or pathways involved in T-DNA integration.

Introduction

Genetic transformation of plants by *Agrobacterium* involves the transfer of T-DNA from its tumor-inducing plasmid to the host cell nuclear genome. In this way, *Agrobacterium* has provided us with a means to produce genetically modified plants. T-DNA is transferred as a single stranded molecule (T-strand) from the bacteria to the plant cell nucleus. During this process, several *Agrobacterium* Vir proteins accompany the T-strand. The T-DNA integrates at a random position in the nuclear genome of the plant cells, but the precise mechanism of T-DNA integration into the plant genome remains unclear. Two possible models for T-DNA integration have been proposed over the years (see for reviews Tzfira *et al.* 2004; Gelvin 2010). In the strand-invasion model, T-DNA integration depends on the microhomology between T-strand and plant DNA sequences. It was suggested that single stranded DNA is preferential for integration (Rodenburg *et al.* 1989; Gheysen *et al.* 1991; Mayerhofer *et al.* 1991). In the DNA double strand break repair model, the T-strand is first converted into double stranded DNA and then this is integrated into a double strand break site in the genome. The second model was supported by the fact that DSBs are the preferential targets for T-DNA integration (Salomon and Puchta 1998; Chilton and Que 2003; Tzfira *et al.* 2003).

Using yeast as a model it was shown in our lab that random T-DNA integration in yeast (*Saccharomyces cerevisiae*) is dependent on the non-homologous end joining (NHEJ) pathway of DSB repair, and that proteins such as Ku70, Ku80 and DNA ligase IV are essential for T-DNA integration (van Attikum *et al.* 2001). Inactivation of the NHEJ pathway still allowed integration via homologous recombination provided that the T-DNA carried an area of homology with the yeast genome (van Attikum and Hooykaas 2003). In this way gene targeting could be facilitated in yeasts and fungi (Kooistra *et al.* 2004). Many proteins involved in the NHEJ pathway are conserved in plants, including Ku70, Ku80 and Lig4 and therefore attempts were made to facilitate gene targeting in plants by inactivating NHEJ in plants. However, studies on T-DNA integration with Arabidopsis NHEJ mutants gave variable results. Initial publications reported about strongly or mildly decreased stable transformation in Arabidopsis *ku70* and *ku80* mutants (Friesner and Britt 2003; Li *et al.* 2005; Jia *et al.* 2012; Mestiri *et al.* 2014), whereas a more recent publication even reported increased T-DNA integration in such mutants (Park *et al.* 2015). A decrease in floral dip transformation was reported for a Arabidopsis *lig4* mutant (Friesner and Britt 2003), but others found no alteration in the frequency of stable transformation in both floral dip and the root tumorigenesis assay (van Attikum *et al.* 2003; Jia *et al.* 2012; Park *et al.* 2015). Therefore, in plants T-DNA integration must be possible by another pathway. In animal and plant cells in the absence of c-NHEJ, DSB repair is possible by backup pathways (b-NHEJ) that so far are not fully characterized and these may play a role in T-DNA integration.

In the b-NHEJ repair pathway in animal cells, Parp1 has been identified to play an essential role together with XRCC1, DNA ligase III, Mre11, as well as other end processing proteins (Audebert *et al.* 2004; Cheng *et al.* 2011). Poly(ADP-ribose) polymerases (PARPs) are abundant nuclear enzymes that have been implicated in many cellular processes in higher eukaryotes, including stress responses, mitosis, transcription and DNA repair (Schreiber *et al.* 2006). Three Parp proteins have been found to be activated in response to DNA damage in animals: Parp1, Parp2 and Parp3 (Gibson and Kraus 2012). Parp1 and Parp2 are involved in

the DNA damage response (DDR), DNA single strand break repair (SSBR) and base excision repair (BER) (Beck, Robert, *et al.* 2014). Parp3 was only recently discovered as a factor recruited to DNA damage sites and which accelerates non-homologous end-joining probably by its interaction with the components of the c-NHEJ pathway, including Ku70/Ku80 and APLF proteins (Boehler et al. 2011; Beck et al. 2014). Thus, Parp3 is thought to be involved in DSB repair via c-NHEJ.

In plants, XRCC1 has been identified to play an important role in NHEJ in the absence of Ku (Charbonnel *et al.* 2010). Our previous work showed that the Arabidopsis Parp1 and Parp2 are involved in a b-NHEJ pathway, called MMEJ (microhomology-mediated end-joining) which uses microhomology for repair (Jia *et al.* 2013). Triple mutants inactivating both c-NHEJ and b-NHEJ were constructed in our lab. This included the triple mutant *ku80parp1parp2* which was hypersensitive to DNA damage, but still conferred T-DNA integration (Jia, 2011). These results suggest that there may be further redundancy or still other factors are involved in T-DNA integration. Recently, the *PARP3* gene was discovered in plants (Rissel *et al.* 2014; Stolarek *et al.* 2015). Its activity might explain why there was still T-DNA integration in the *ku80parp1parp2* triple mutant.

In order to get a better understanding of NHEJ repair pathways and *Agrobacterium*-mediated T-DNA integration in plants, mutants in either c-NHEJ, b-NHEJ or both pathways were tested in root transformation assays. In order to extend our collection of NHEJ mutants, homozygous *parp3* and *xrcc1* mutants were isolated and characterized. The *parp3* mutant was crossed with *parp1* and *parp2* mutants to obtain the homozygous triple mutant of *parp1parp2parp3* (*p1p2p3*). The single, double and triple mutants were tested for the sensitivity to DNA damaging agents and in a root transformation assay. The *xrcc1* mutant was crossed with *ku80* to obtain the *ku80xrcc1* double mutant. Together with other NHEJ mutants including *ku80*, *ku70*, *xrcc1*, *lig4*, *lig6*, *lig4lig6*, and *ku80p1p2*, the *p1p2p3* and *k80xrcc1* mutant lines were also analyzed for *Agrobacterium*-mediated T-DNA integration in root transformation assays.

Materials and methods

Plant material

The *Atparp3* (At5g22470; SALK_108092) and *Atxrcc1* (At1g80420; SALK_125373) T-DNA insertion lines, ecotype Col-0, were obtained from the SALK collection (Alonso *et al.* 2003). The *Atmre11-2* (Bundock and Hooykaas 2002), *Atparp1* (GABI-Kat Line 692A05) (Jia *et al.* 2013), *Atparp2* (SALK_640400) (Jia *et al.* 2013), *Atlig4* (SALK_044027), *Atku70* (SALK_123114) and *Atku80* (SALK_016627) (Jia *et al.* 2012) and *Atlig6* (SALK_065307) (Jia, 2011) mutants have been previously described.

Characterization of the Atparp3 and Atxrcc1 mutants

DNA was extracted from individual plants using the CTAB DNA isolation protocol. The T-DNA insertion sites of the *parp3* and *xrcc1* mutants were mapped with a gene-specific primer (SP539 or SP544 for *parp3*; SP170 or SP171 for *xrcc1*) and a T-DNA specific primer (LBa1 or RB) and PCR products were cloned and sequenced. 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. The sequences of all the primers are listed in Table1. For Southern blot analysis, 10 µg DNA from the mutants were digested with *Hin*dIII and separated 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.

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

Seeds of wild-type, *parp1, parp2, parp3, p1p2, p1p3, p1p2p3, ku80, xrcc1, ku80xrcc1* mutants were surface-sterilized as described (Weijers *et al.* 2001) and germinated on solid ½ MS medium without additions or containing 0.02 µg/ml bleomycin (Sigma), 0.05 µg/ml bleomycin, 0.005% (v/v) MMS (Sigma) or 0.007% MMS. After 2 weeks the mutants photographed.

Root transformation and GUS assays

Root transformation were performed as described (Vergunst *et al.* 2000). Root segments were infected with *A.tumefaciens* LBA1100 harboring the binary vector pCambia3301. The T-DNA from pCambia3301 contains a phosphinothricin selection cassette and a GUS gene. After co-cultivation on callus induction medium containing 100 µM acetosyringone for 48 hours, root segments were washed, dried and incubated on shoot induction medium plus phosphinothricin 30 µg/ml, 500 µg/ml carbenicillin and 100 µg/ml vancomycin. After 3-4 weeks, plates were photographed and transformation efficiencies was scored as infected root segments that produced any form of callus.

For transient GUS activity assays, root segments were washed after 72 hr cocultivation, dried and stained with X-Gluc overnight at 37°C. Root segments were destained with 70% ethanol and visualized using a microscope.

For quantification of GUS activity, root segments were washed, dried and disrupted to a powder under liquid N2 in a TissueLyser (Retch, Haan, Germany). Protein extraction buffer (1x Na-phosphate/EDTA buffer PH 7.0, 0.1% sodium lauryl sarcosine (SLS), 0.1% Triton-X100, 10 mM β-mercaptoethanol) was added to tissue powder. Soluble protein was isolated by centrifugation at 4°C. The protein concentration was determined by using the BIO-RAD protein assay reagent. Ten µl protein extracts in duplicate were co-cultivated in 190 µl extraction buffer/MUG at 37°C. The fluorescence value was scored after 0.5, 1, 3, 5 hours by a Perkin Elmer 1420 Fluorimeter. GUS activities from triplicate transformations were normalized against total protein content to correct for differences in protein extraction efficiencies. Statistical analyses were performed using Prism version 5 (GraphPad Software Inc.).

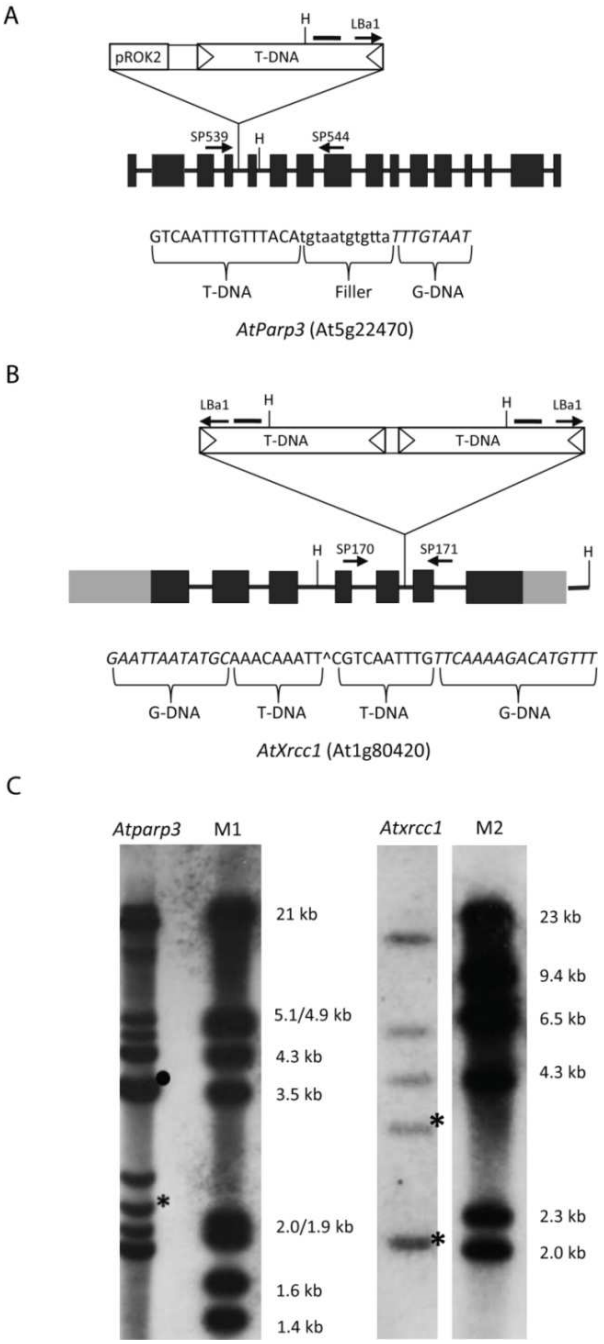

Figure 1. Molecular analysis of the T-DNA insertions in the *PARP3* and *XRCC1* loci. Genomic organization of the *PARP3* (**A**) and *XRCC1* (**B**) locus is indicated with the positions of the inserted T-DNA. Exons are shown as black boxes, 3' and 5' UTRs are shown as gray boxes and introns are shown as lines. The probe (▬) and the restriction enzyme digestion site (H: *Hin*dIII) used for Southern blot analysis, are indicated. Genomic DNA sequences (gDNA) flanking the T-DNA insertion are shown in italics. T-DNA border indicated with triangle. **(C)** Southern blot analysis of the *parp3* and *xrcc1* T-DNA insertion mutant. The genomic DNA was digested by *Hin*dIII. M1, M2: Lambda DNA *Eco*RI+*Hin*dIII, Lambda DNA *Hin*dIII marker. The expected bands of 2174 bp (*parp3* mutant), 2173 bp and 3340 bp (*xrcc1* mutant) are indicated with an asterisk. Tandem repeat of 3.6 kb is indicated with a dot.

Results

Identification of the parp3 and xrcc1 mutants

Previously it was found that in the triple *ku80p1p2* mutant inactivating both c-NHEJ and b-NHEJ at the same time, T-DNA integration was still observed. Recently, a third *PARP* gene was discovered in plants called *PARP3* (Rissel *et al.* 2014). As *PARP3* is possibly redundant to *PARP2* and *PARP1*, we isolated and characterized the *parp3* homozygous mutant and crossed it with *p1p2* (**Figure 1**). Homozygous mutants were identified by using T-DNA specific primers for the left border or right border in combination with gene-specific primers flanking the insertion site. No PCR products were obtained for homozygous mutants using two gene-specific primers. The insertion site of the T-DNA was mapped by sequencing. The T-DNA was integrated in the *PARP3* gene in intron 4, whereby 12 bp filler DNA was inserted at the LB end (**Figure 1A**). The RB was integrated with part of the original pROK2 vector. Therefore, the RB integration site could not be mapped. For Southern blotting, genomic DNA of the *parp3* mutant was digested with *Hin*dIII. A diagnostic band of 2174 bp was expected connecting the T-DNA with the *PARP3* gene, which can indeed be seen on the gel (**Figure 1C**). Besides the 2 kb band extra bands were detected, indicating that additional T-DNAs were randomly integrated in the genome of the *parp3* mutant. The band around 3.5 kb indicated a random T-DNA integration as a tandem repeat.

Another protein that seems important for b-NHEJ is XRCC1 (Charbonnel *et al.* 2010). The homozygous mutant was isolated and characterized. The results showed that two T-DNAs were inserted in intron 5 in inverted orientation, with the LBs flanking the plant DNA and 3 bp of the plant DNA missing as well as the LB sequence of one of the T-DNAs (**Figure 1B**). For Southern blotting genomic DNA was again digested with *Hin*dIII. Two bands of 2173 bp and 3343 bp were expected diagnostic for the connection between the T-DNAs and the *XRCC1* gene. These can indeed be seen on the gel (**Figure 1C**). Besides, extra bands were detected, indicating that additional T-DNAs were randomly integrated in the genome of the *xrcc1* mutant.

DNA damage response

In order to study whether Parp3 functions in DNA repair, the *parp3* mutant was tested for sensitivity to the genotoxic agents bleomycin and MMS. As no difference was seen with the wildtype, *p1p3* double and *p1p2p3* triple mutants were obtained by crossing the individual mutants and assayed in the same way for hypersensitivity to genotoxic agents as this may reveal gene redundancy. Without any treatments, *p1p2* (Jia *et al.* 2013), *p1p3* double and *p1p2p3* triple mutants grew the same as wild type. When treated with bleomycin, there were again no clear differences in growth seen between the *parp* mutants and wild type plants (data no shown). However, as can be seen in **Figure 2A**, when treated with MMS, the *p1p2p3* triple and *p1p3* double mutants showed somewhat more sensitivity than each of the single mutants, especially when treated with 0.005% MMS. This result suggests that Parp3 is indeed involved in DNA repair and even may play a more important role than Parp2 in the repair of MMS damage.

The *xrcc1* mutant was treated in a similar way to test its function in DNA repair (**Figure 2B**). As no difference was observed with the wild type, a *ku80xrcc1* double mutant was

obtained by crossing the single mutants and assayed in the same way for hypersensitivity to genotoxic agents (**Figure 2B**). In normal growth conditions, the *ku80xrcc1* double mutant grew the same as the wild type and single mutants. When treated with bleomycin, the *ku80xrcc1* double mutant showed the same sensitivity as the *ku80* single mutant. However, the *ku80xrcc1* mutant showed more sensitivity to MMS than the wildtype and each of the single mutants. These results indicate that XRCC1 and Ku80 repair MMS induced damage by different pathways.

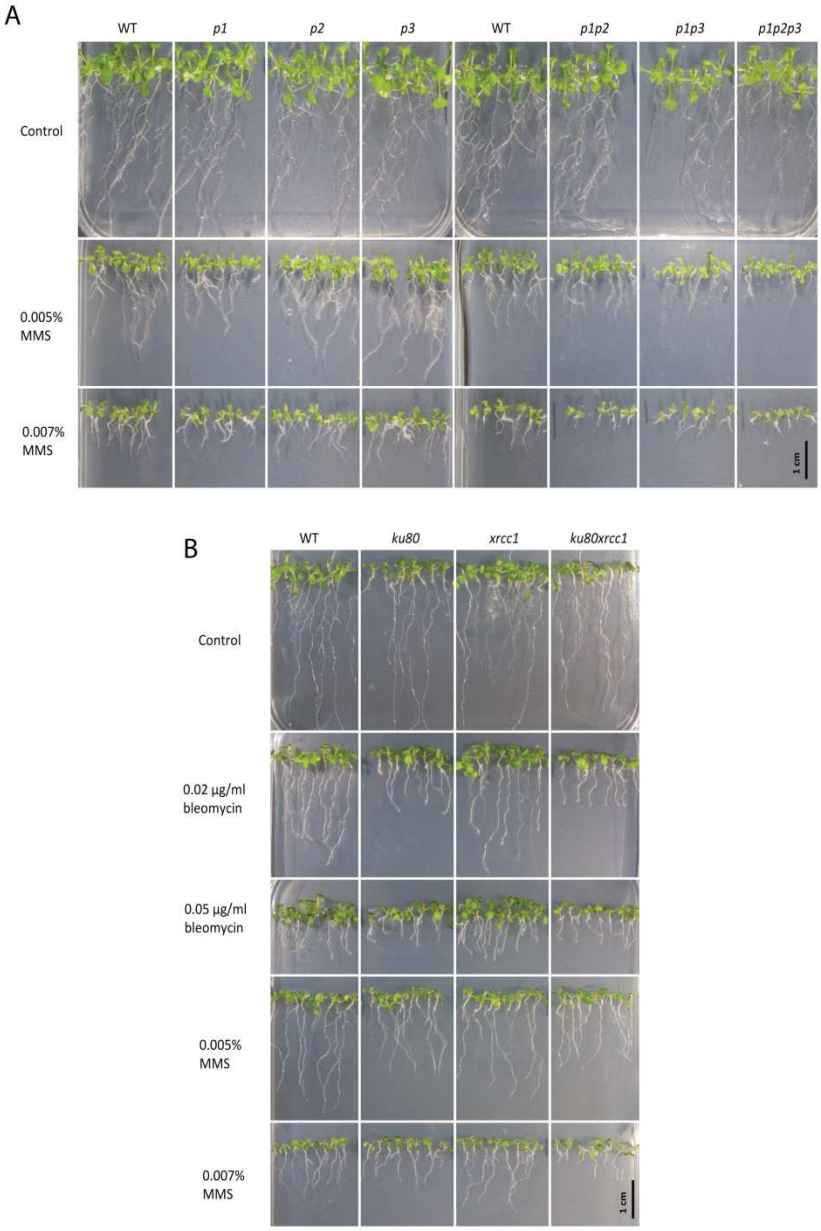

Figure 2. NHEJ mutants response to DNA damaging treatments. (**A**) Phenotypes of wild-type plants and *parp1*, *parp2*, *parp3*, *p1p2*, *p1p3* and *p1p2p3* mutants germinated on ½ MS medium (control) or ½ MS medium containing 0.005% and 0.007% MMS photograph taken 2 weeks after germination. (**B**) Phenotypes of wild-type plants and *ku80*, *xrcc1* and *ku80xrcc1* mutants germinated on ½ MS medium (control) or ½ MS medium containing 0.02 µg/ml, 0.05 µg/ml bleomycin or 0.005%, 0.007% MMS photograph taken 2 weeks after germination.

Root transformation

In order to determine the effects of the mutations on Agrobacterium T-DNA integration in somatic plant cells, we performed a root transformation assay using Agrobacterium strain LBA1100 harboring the binary vector (pCambia3301) and selected for callus formation in the presence of phosphinothricin (ppt). Roots of WT, *ku80*, *ku70*, *parp1*, *parp2*, *parp3*, *xrcc1*, *lig4*, *lig6*, *ku80xrcc1*, *lig4lig6*, *p1p2*, *p1p2p3* and *ku80p1p2* mutant lines were co-cultivated with the Agrobacterium strain, and numbers of green calli were counted after 4 weeks. Longer culture led to green shoot formation from these calli. Data from three independent tests and more than 300 root segments of each genotype are presented in **Figure 3**. Roots from *ku80*, *ku70*, *parp1*, *parp2*, *parp3*, *xrcc1*, *lig6*, *p1p2* and *p1p2p3* mutants were transformed as well as the wild type. The double mutant *ku80xrcc1* and triple mutant *ku80p1p2*, which were supposed to be disturbed in both c-NHEJ and b-NHEJ repair pathways, gave significantly less transformed calli than the wild type roots, indicating that NHEJ repair pathways are partly responsible for the T-DNA integration process. Interestingly, roots from the *lig4* mutant produced significantly more transformed calli than roots from wild type plants, indicating that Lig4 is not required for Agrobacterium T-DNA integration in plants and may even be somewhat inhibitory to the T-DNA integration process. However, the roots of the *lig4lig6* double mutant produced the same number of transformed calli as roots from wild-type plants.

Transient transformation is not affected in NHEJ mutants

We expected that the mutation of NHEJ genes would not affect the entry of T-DNA into the plant cell nucleus. To test this, we infected root segments of wild type and Arabidopsis mutant lines with the same Agrobacterium strain LBA1100 harboring the binary vector pCambia3301 which contains a CaMV 35S promoter *gusA*-intron gene. This *gusA* gene allows expression of GUS activity in plant cells, but not bacteria. After inoculation with Agrobacterium and co-cultivation for 3 days, the root segments were stained with X-Gluc to reveal transient transformation. As shown in **Figure 4A**, most root segments were stained with blue color, but this was difficult to quantify. In order to quantify the GUS activity, we used the fluorescent β-glucuronidase substrate MUG (4-methylumbelliferyl-β-d-galactopyranoside) instead. Three days after infection, proteins were extracted from root segments and tested in this way for GUS activity. As seen in **Figure 4B**, no significant differences were observed in the GUS activity between wild type and NHEJ mutants roots co-cultivated with Agrobacterium. Thus, NHEJ mutations that increased or reduced stable transformation frequencies did not affect T-DNA transfer and transient expression of the T-DNA.

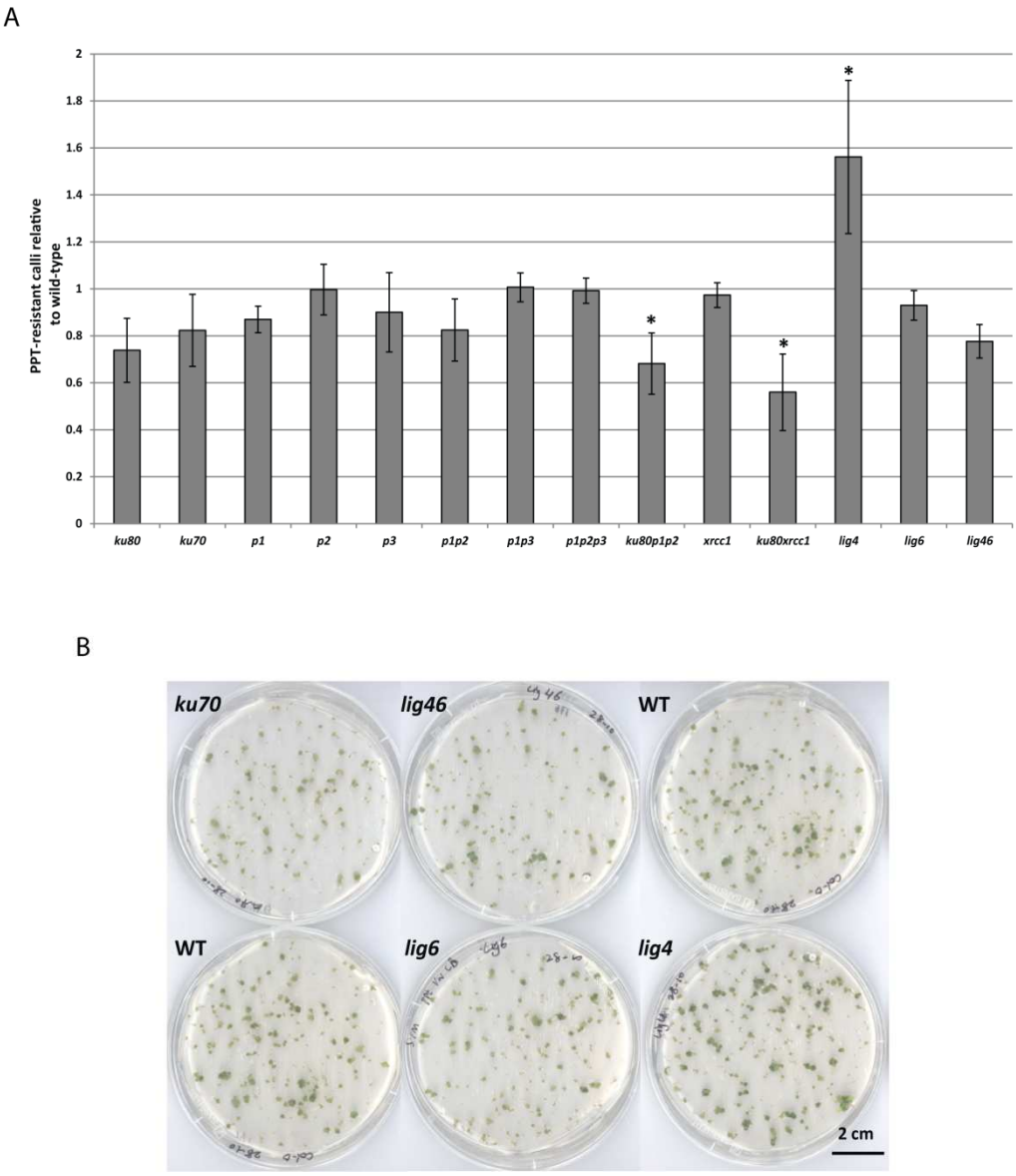

Figure 3. Stable root transformation of Arabidopsis NHEJ mutants. (**A**) Root segments from wild-type and mutant plants were co-cultivated with Agrobacterium stain LBA1100 harboring pCambia3301 for 48 hours, and transferred to selection induction medium. Efficiency of transformation as represented by the ratio of mean number of green calli per root segment relative to wild type. Asterisk indicates a significant difference relative to wild-type plants (ANOVA, $P<0.05$). (**B**) Examples of root transformation assays from wild-type and *ku80*, *lig4*, *lig6*, *lig46* mutants. Photographs were taken 4 weeks after co-cultivation.

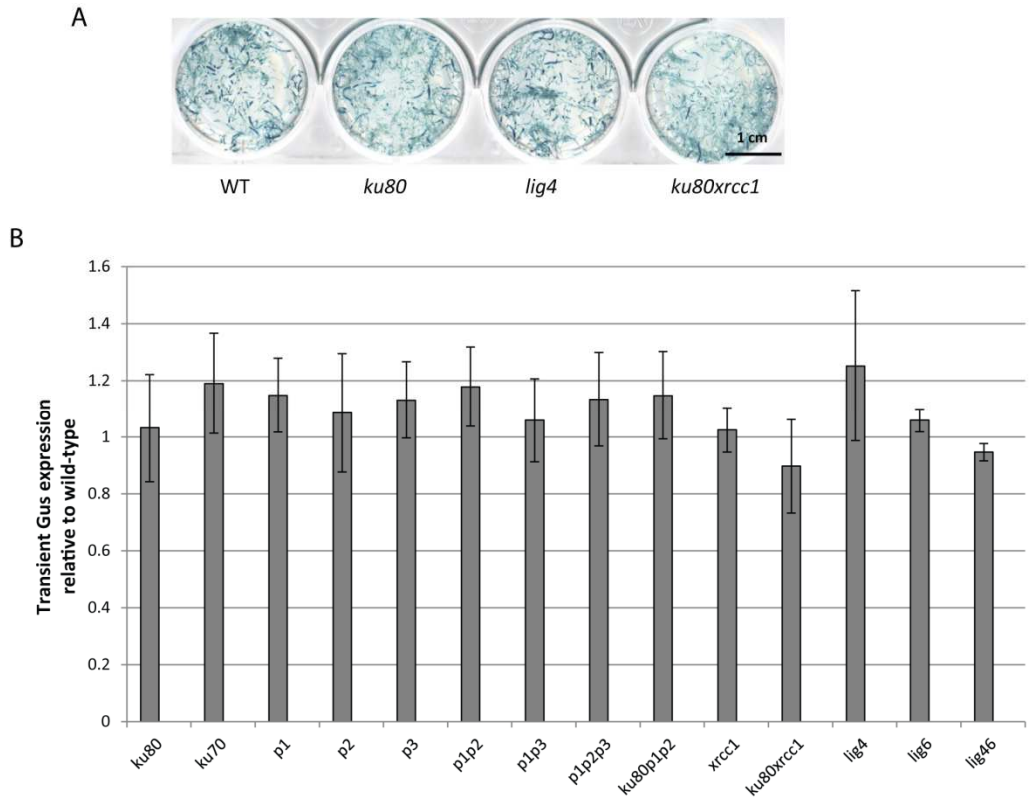

Figure 4. Transient GUS assay of Arabidopsis NHEJ mutants. (**A**) Root segments from wild-type and mutant plants were co-cultivated with Agrobacterium stain LBA1100 harboring pCambia3301 for 72 hours, and then stained with X-Gluc. (**B**) The transient GUS expression level is quantified by MUG assay and presented as the ratio of the level of MUG activity relative to wild type. Statistics analysis showed no significant differences.

Discussion

Our previous work showed that NHEJ can still occur in *parp1parp2* and *ku80parp1parp2* mutants (Jia *et al.* 2013), suggesting that there may be a third pathway of NHEJ in plants or that there may still be functionally redundant proteins taking over the function of Parp1 and Parp2 when they are absent. The recently discovered *PARP3* gene might represent such a redundant protein. The mammalian *PARP3* gene has been reported to be involved in the DNA damage response and to interact with different partners belonging to the c-NHEJ pathway. Therefore, a T-DNA insertion mutant of *PARP3* was obtained and characterized. There was no phenotypical difference between the *parp* single mutants and the wild-type plants under normal growth conditions or after genotoxic treatment. However, the *p1p3* double and *p1p2p3* triple mutants showed more sensitivity to MMS. It means that Parp3 is indeed involved in DNA repair in plants. Further work is needed to find out to what extent Parp1, Parp2 and Parp3 are functionally redundant in the same NHEJ repair pathway in plants. Recently, unexpectedly Parp1 was shown to be involved not only in b-NHEJ (Beck, Robert, *et al.* 2014), but also in c-NHEJ in mammalian cells as a recruitment factor for the chromatin remodeler CHD2 (Luijsterburg *et al.* 2016).

A

Floral dip transformation efficiency

	AGL1/pSDM3834								AGL1/pSDM3900			
	Col-o	*ku80*	*parp1*	*xrcc1*	*ku80parp1*	*Ws*	*mre11-2(Ws)*	*ku70(Ws)*	*Col-o*	*ku80*	*parp1*	*xrcc1*
Mean	10.2	2.2	10.8	13.8	4	20.6	4.4	5	30.3	5.5	28.3	41.9
S.D	4.02	1.47	3.76	3.43	2.28	3.44	0.80	2.68	6.18	1.91	3.49	8.98
N.	5	5	5	5	5	5	5	5	10	10	10	10

B

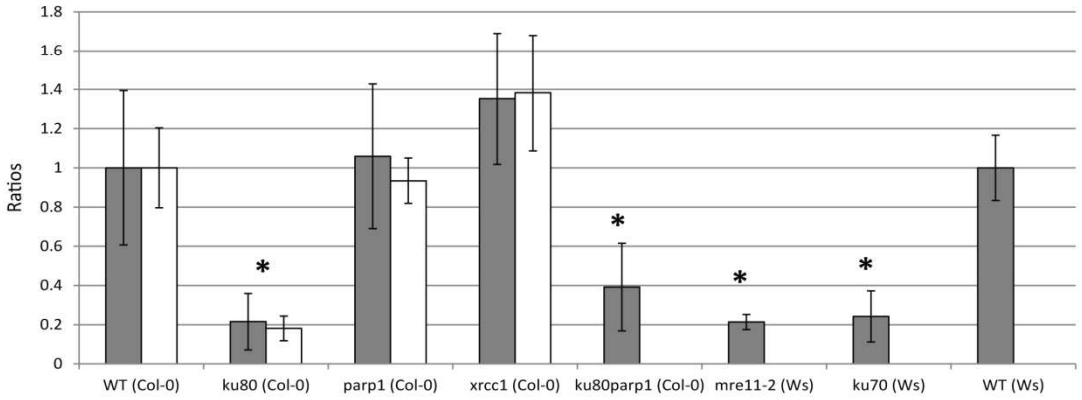

Figure 5. Transformation frequencies using floral dip assay. One or half gram of seeds from the wild-type (Col-0 and Ws) and the NHEJ mutants obtained after floral dip transformations were selected on hygromycin (for pSDM3834) or phosphinothricin (for pSDM3900). The number of selection-marker-resistant seedlings was scored 2 weeks after germination. Plates with contamination were excluded, and mean numbers of resistant seedling (per plate), standard errors and numbers of measurements (N) are shown in **(A)**. **(B)** The transformation efficiency is presented as the ratio of the number of selection-marker-resistant seedlings in the mutants and wild-type. The grey bar indicates the data for pSDM3834, and the white bar represents pSDM3900. Asterisk indicates a significant difference relative to wild-type plants (ANOVA, *P<0.001*).

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

Name	Locus	Sequence
LBa1	T-DNA left end	5'-TGGTTCACGTAGTGGGCCATCG-3'
RB	T-DNA right end	5'-TTTGGAACTGACAGAACCGC-3'
Sp170	*Atxrcc1*	5'-GACACTCTAAAGAAACGTTCC-3'
Sp171	*Atxrcc1*	5'-GAATCTCCGTTTTAACCATCC-3'
Sp271	pROK2	5'-CCCGTGTTCTCTCCAAATG-3'
Sp272	pROK2	5'-CAGGTCCCCAGATTAGCC-3'
Sp539	*Atparp3*	5'-GTGAGTGGTGCAGTTGCGTGT-3'
Sp544	*Atparp3*	5'-CTTCGGCATTAGGGTCATCTC-3'

In this work also a mutant with a T-DNA insertion in the *XRCC1* gene was isolated and characterized. The Arabidopsis *xrcc1* mutant has been shown to be hypersensitive to γ-radiation and even more sensitive than *ku80* mutant plants (Charbonnel *et al.* 2010). However, we saw no phenotypical difference between *xrcc1* mutant and wild-type plants after treated with MMS or bleomycin agents. Double mutant *ku80xrcc1* plants showed similar or increased sensitivity compared to the *ku80* single mutant to a number of different genotoxic agents, confirming that XRCC1 is involved in DNA repair pathways in plants, but the precise role in the NHEJ pathways remain to be determined. In human cells, XRCC1 has been shown to play an important role together with DNA Lig3 in b-NHEJ pathways (Della-Maria *et al.* 2011). Due to lack of Lig3 in plants, XRCC1 must act in a different manner during the b-NHEJ repair in plants, which may depend on poly (ADP-ribose) synthesis (Breslin *et al.* 2015).

Agrobacterium T-DNA molecules integrate into plant DNA double strand break sites (Tzfira *et al.* 2004). Thus, double strand break repair mechanisms are hypothesized to be involved in the integration of Agrobacterium T-DNA in plants. Previously, our group had shown that non-homologous T-DNA is integrated by NHEJ in yeast, and that NHEJ proteins including the Ku70/Ku80 and Lig4 play an essential role in T-DNA integration in yeast. Several studies have investigated the role of NHEJ in *Agrobacterium*-mediated T-DNA integration in plants, but the results obtained so far are variable (Friesner and Britt 2003; van Attikum *et al.* 2003; Gallego *et al.* 2003; Li *et al.* 2005; Jia *et al.* 2012; Nishizawa-Yokoi *et al.* 2012; Mestiri *et al.* 2014; Park *et al.* 2015). The inconsistency of these findings is probably due to using different mutant lines or different experimental procedures. In order to test whether NHEJ proteins are involved in T-DNA integration, we performed root transformation assays. Although a lower T-DNA integration frequency was observed in the *ku80p1p2* and *ku80xrcc1* mutants, these mutants were still able to stably integrate T-DNA. In addition the *ku80p1p2* mutant can still repair nuclease-induced DSBs (**Chapter 5**) indicating that either another redundant protein is present or other repair pathways are active.

The root transformation frequency in *ku80* and *ku70* c-NHEJ mutants did not show significant differences compared to the wild type. However, our previous results from floral dip transformations with NHEJ mutants showed that the transformation frequency is highly reduced in *ku80*, *ku70* and *mre11-2* mutants (**Figure 5**). This may be due to differences of target tissues. Floral dip results with *parp1*, *xrcc1* and *ku80parp1* mutants showed that PARP1 and XRCC1 has no essential function in floral dip transformation (**Figure 5**). Roots and other somatic cells are the natural target for *Agrobacterium*-mediated transformation, whereas the target cells in floral dip transformation are the female gametophytes.

Our results of root transformation showed that mutations in either the c-NHEJ or b-NHEJ pathway did not significantly impact T-DNA integration, but mutations in both pathways together significantly reduced root transformation efficiency. Other reports similarly showed that T-DNA integration was not completely suppressed or not decreased at all in *ku80parp1* and *ku80xrcc1* double mutants (Mestiri *et al.* 2014; Park *et al.* 2015). It could be that c-NHEJ and b-NHEJ are functionally redundant in T-DNA integration. Mestiri *et al.* (2014) observed an about three-fold decrease in both floral dip transformation and root transformation frequencies of single mutants in the b-NHEJ pathway. However, the results

from Park *et al.* (2015) indicated that c-NHEJ and b-NHEJ proteins do not positively contribute to transformation susceptibility and may even limit stable transformation. One possibility is that different experimental conditions (such as Agrobacterium inoculation concentrations) caused different observations. Besides, Arabidopsis mutant lines may show altered growth or developmental characteristics, which may also affect the outcomes of transformations.

Although conflicting results were obtained from several research groups including our own investigating the role of NHEJ proteins in *Agrobacterium*-mediated plant transformation, all these findings show at least that disruption of one or multiple NHEJ repair pathways does not eliminate transformation, suggesting that another DNA repair pathway is involved in T-DNA integration. A recent study showed that the *Arabidopsis* Pol θ ortholog *Tebichi* (*Teb*) is essential for T-DNA integration (Kregten et al, 2016). Since mutations in c-NHEJ components did show decreased transformation frequencies, they probably function together with Pol θ during the integration process.

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