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# *Agrobacterium* T-DNA integration into the plant genome can occur without the activity of key non-homologous end-joining proteins

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## SUMMARY

**Non-homologous end joining (NHEJ) is the major model proposed for *Agrobacterium* T-DNA integration into the plant genome. In animal cells, several proteins, including KU70, KU80, ARTEMIS, DNA-PKcs, DNA ligase IV (LIG4), Ataxia telangiectasia mutated (ATM), and ATM- and Rad3-related (ATR), play an important role in 'classical' (c)NHEJ. Other proteins, including histone H1 (HON1), XRCC1, and PARP1, participate in a 'backup' (b)NHEJ process. We examined transient and stable transformation frequencies of *Arabidopsis thaliana* roots mutant for numerous NHEJ and other related genes. Mutants of *KU70*, *KU80*, and the plant-specific *DNA LIGASE VI* (*LIG6*) showed increased stable transformation susceptibility. However, these mutants showed transient transformation susceptibility similar to that of wild-type plants, suggesting enhanced T-DNA integration in these mutants. These results were confirmed using a promoter-trap transformation vector that requires T-DNA integration into the plant genome to activate a promoterless gusA (*uidA*) gene, by virus-induced gene silencing (VIGS) of *Nicotiana benthamiana* NHEJ genes, and by biochemical assays for T-DNA integration. No alteration in transient or stable transformation frequencies was detected with *atm*, *atr*, *lig4*, *xrcc1*, or *parp1* mutants. However, mutation of *parp1* caused high levels of T-DNA integration and transgene methylation. A double mutant (*ku80*/*parp1*), knocking out components of both NHEJ pathways, did not show any decrease in stable transformation or T-DNA integration. Thus, T-DNA integration does not require known NHEJ proteins, suggesting an alternative route for integration.**

**Keywords: *Agrobacterium*, non-homologous end joining, T-DNA integration, plant transformation, *Arabidopsis thaliana*, *Nicotiana benthamiana*.**

## INTRODUCTION

Members of the genus *Agrobacterium*, which cause crown gall, cane gall, and hairy root disease, are the only known organisms that naturally transfer DNA to plants. Transferred DNA (T-DNA) enters the plant as a single-stranded molecule (Stachel *et al.*, 1986; Tinland *et al.*, 1994; Yusibov *et al.*, 1994) that may eventually integrate into the nuclear genome. Although T-DNA integration is random (Kim *et al.*, 2007), the mechanism of integration remains unclear. In plants, T-DNA integration does not use homologous recombination, although microhomology between T-DNA and chromosomal integration sites may occur. The discovery of such microhomologies engendered a 'single-strand invasion' model (Mayerhofer *et al.*, 1991), by which T-DNA integrates into plant DNA by illegitimate recombination. However, the presence of integrated T-DNA head-to-head dimers suggested that double-stranded (ds) DNA breaks, followed by non-homologous end joining (NHEJ) of double-stranded T-DNA to plant

DNA, occurs. Irradiated protoplasts show a higher DNA integration frequency than do non-irradiated protoplasts (Köhler *et al.*, 1989), suggesting that dsDNA damage sites could be targets of T-DNA integration. Indeed, T-DNA molecules preferentially integrate into dsDNA break sites (Salomon and Puchta, 1998; Chilton and Que, 2003; Tzfira *et al.*, 2003); see Tzfira *et al.* (2004) for T-DNA integration models.

Environmental stress and normal metabolic processes can cause DNA damage in plants (Britt, 1996). NHEJ is a major pathway for the repair of dsDNA breaks, requiring little or no DNA sequence homology at the damaged ends. Studies in mammalian cells and yeast have identified components of both 'classical' and 'backup' NHEJ pathways (Mladenov and Iliakis, 2011; Scott and Pandita, 2006; Decottignies, 2013). Plants contain many of these proteins important for NHEJ. These include, for cNHEJ, the signaling proteins ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR), and proteins directly involved in dsDNA break repair, including KU70, KU80, X-ray repair cross-complementing protein 4 (XRCC4), and DNA LIGASE IV (LIG4; Bleuyard *et al.*, 2006). bNHEJ uses histone H1 (HON1), XRCC1, and poly(ADP-ribose) polymerases (PARP). HON1 may play a role similar to that of the KU proteins by recognizing double-stranded (ds) DNA breaks, whereas PARP1 may play numerous roles by modifying HON1 and by interacting with and inactivating DNA methyltransferases, resulting in DNA hypomethylation (Zardo *et al.*, 1999; Huber *et al.*, 2004; Althaus, 2005; Reale *et al.*, 2005; Woodhouse *et al.*, 2008; Caiafa *et al.*, 2009; Mladenov and Iliakis, 2011; Ciccarone *et al.*, 2012; Zampieri *et al.*, 2012). When previously examined, the role of these proteins in T-DNA integration is controversial. van Attikum *et al.* (2001) showed that KU70- and LIG4-defective yeast strains integrate T-DNA poorly. However, *LIG4* mutation had either little (Friesner and Britt, 2003) or no effect on plant transformation (van Attikum *et al.*, 2003). Similarly, *ku80* mutant Arabidopsis plants and *ku70*, *ku80*, and *lig4* deficient rice showed different transformation responses in different studies (Friesner and Britt, 2003; Gallego *et al.*, 2003; Li *et al.*, 2005; Jia *et al.*, 2012; Nishizawa-Yokoi *et al.*, 2012). Recently, Mestiri *et al.* (2014) showed that mutations simultaneously eliminating numerous DNA repair pathways resulted in lowered transformation frequency; however, simultaneous disruption of all known pathways still allowed some level of transformation, suggesting that some yet unknown pathway for T-DNA integration exists. In order to reconcile these disparate results, and to understand better the role of NHEJ proteins in *Agrobacterium*-mediated plant transformation, we examined root transformation frequencies of mutant Arabidopsis plants, or used virus-induced gene silencing (VIGS) to decrease expression of targeted NHEJ proteins in *Nicotiana benthamiana.* Surprisingly, we discovered that reduction of many cNHEJ proteins increased T-DNA integration and transformation efficiency, whereas disruption of *PARP1* increased T-DNA integration (insertion of T-DNA into the plant genome) without a concomitant increase in stable transformation efficiency (phenotypes associated with stable expression of T-DNA-encoded transgenes). Simultaneous disruption of both NHEJ pathways did not decrease either stable transformation or T-DNA integration, indicating that yet other pathways for T-DNA integration exist.

## RESULTS

### Arabidopsis *ku80*, *ku70*, and *lig6* mutants are hypersusceptible to root transformation

We conducted quantitative stable and transient root transformation assays (Zhu *et al.*, 2003) to determine the importance of cNHEJ (KU80, KU70, ATM, ATR, and LIG4), bNHEJ (PARP1 and XRCC1) and other (DNA LIGASE VI; LIG6) NHEJ-related proteins for *Agrobacterium*-mediated transformation. Roots are the natural target for *Agrobacterium*-mediated transformation, and behave differently in transformation assays (Mysore *et al.*, 2000a) than do Arabidopsis floral tissues or rice calli used by others to study NHEJ (van Attikum *et al.*, 2003; Friesner and Britt, 2003; Gallego *et al.*, 2003; Li *et al.*, 2005; Jia *et al.*, 2012; Nishizawa-Yokoi *et al.*, 2012). We used homozygous Arabidopsis mutants containing T-DNA insertions in genes encoding these proteins. RT-PCR analysis of root RNA from these mutants revealed no full-length transcripts for these NHEJ genes (Figure S1), and the tested cNHEJ mutants were hypersensitive to bleomycin, a radiomimetic drug which induces dsDNA breaks (Figure S2).

Figures 1(a) and S3(a) show the results of stable root transformation assays for cNHEJ mutants, using the oncogenic strain *A. tumefaciens* A208 and selecting for tumors. *ku70*, *ku80*, and *lig6* mutants (Col-0 background) were 2- to 3.5-fold more susceptible to transformation than were wild-type plants. A *ku80* mutant in the Ws background (Li *et al.*, 2005) displayed 1.5-fold increased transformation susceptibility. We have previously shown that roots of wild-type Col-0, and Ws plants respond differently to *A. tumefaciens* A208 (Zhu *et al.*, 2003). Thus, mutation of *ku80* should not necessarily show the same extent of transformation enhancement in the Col-0 and Ws backgrounds. Mutants of *atm*, *atr*, and *lig4* showed transformation frequencies similar to those of wild-type plants. We previously obtained similar results for a different *lig4* mutant (van Attikum *et al.*, 2003). All of these transformation results were independently confirmed in the Gelvin, Mysore, and Zhang laboratories.

We further investigated the effect of bNHEJ mutants on stable root transformation, again using a crown gall tumorigenesis assay. Figure 1(a) shows that mutation of *xrcc1* or *parp1* had no major effect on transformation frequency.

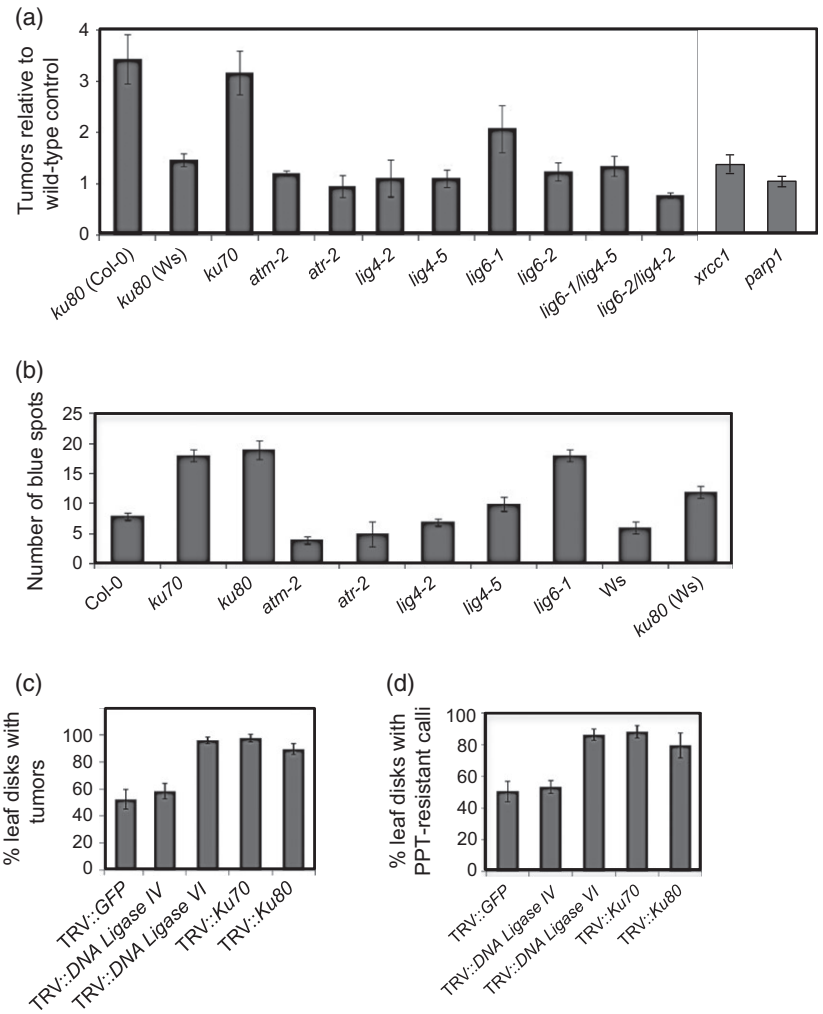


**Figure 1.** Stable transformation of Arabidopsis NHEJ mutants and VIGS-silenced *Nicotiana benthamiana*.

(a) Root segments from wild-type and mutant Arabidopsis plants were inoculated with *A. tumefaciens* A208 and assayed for crown gall tumorigenesis 1 month later. *lig4-2, lig4-5, lig6-1*, and *lig6-2* represent different alleles of *lig4* and *lig6*, respectively. *lig6-1 lig4-5* and *lig6-2 lig4-2* represent double mutants in the Col-0 and Ws backgrounds, respectively (Waterworth *et al.*, 2010). Bacterial inoculation was at $10^6$ cfu $ml^{-1}$. The *Y*-axis indicates the relative number of tumors per 100 root segments compared with the wild-type control.

(b) Root segments from wild-type and mutant Arabidopsis plants were inoculated with an *A. tumefaciens* strain containing the promoter-trap binary vector pKM1. After 15 days, the roots were stained with X-Gluc.

(c) Percentage of leaf disks from VIGS-silenced *N. benthamiana* plants that formed tumors 4 weeks after inoculation by *A. tumefaciens* A348.

(d) Percentage of leaf disks from VIGS-silenced tobacco plants that formed ppt-resistant calli 4 weeks after inoculation by *A. tumefaciens* GV2260(pCAS1). Each bar represents the mean ± standard error (SE) (a, b) or standard deviation (SD) (c, d) from four replicates (20 disks per replicate).

Taken together, these data indicate that no single cNHEJ or bNHEJ protein is required for efficient stable transformation of Arabidopsis roots.

### VIGS of some *N. benthamiana* cNHEJ genes increases transformation susceptibility

To validate the Arabidopsis mutant results in another plant species, we used a VIGS system (Anand *et al.*, 2007) to reduce the expression of cNHEJ genes in *N. benthamiana*. *NbKU70*-silenced plants had shortened internodes (Figure S4a). Figure S4(b) shows that leaves of VIGS-silenced plants contained lowered levels of target RNAs, although silencing of *NbLIG4* was weaker than that of other NHEJ genes studied.

Leaf disks from VIGS-silenced plants were infected with the tumorigenic strain *A. tumefaciens* A348 or, in separate experiments, disarmed *A. tumefaciens* GV2260 containing the binary vector pCAS1. pCAS1 contains a plant-active *bar* gene that confers phosphinothricin (ppt) resistance on stably transformed cells (Nam *et al.*, 1999). Figure 1 shows that silencing of *NbKU70*, *NbKU80*, and *NbLIG6* resulted in leaves with increased susceptibility to tumorigenesis (Figures 1c and S3b) or ppt resistance (Figures 1d and S3c) compared with TRV::*GFP* inoculated control plants. Silencing of *NbLIG4* did not significantly alter transformation susceptibility. These results are similar to those obtained in Arabidopsis. Uninfected leaf disks of all the silenced plants produced calli at an efficiency equal to that of control plants on non-selective callus inducing medium, and showed no observable differences in cell division or proliferation (Figure S5). Taken together, silencing of these cNHEJ genes does not interfere with cellular functions essential for callus cell division.

### NHEJ mutants show no alteration in transient transformation frequency

If NHEJ proteins are required only for T-DNA integration, but not for processes prior to integration, we would expect that mutation of NHEJ genes would not alter susceptibility to transient transformation, which does not require T-DNA

integration (Mysore *et al.*, 1998; Zhu *et al.*, 2003). To test this hypothesis, we infected root segments of wild-type and mutant Arabidopsis plants with *A. tumefaciens* GV3101 harboring the binary vector pBISN1. pBISN1 contains a plant-active gusA-intron gene that does not express β-glucuronidase (GUS) activity in bacteria (Narasimhulu *et al.*, 1996). Six days after infection, we stained the root segments with 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-Gluc) to reveal transient transformation (Nam *et al.*, 1997; Zhu *et al.*, 2003). Figure 2 shows that transient transformation frequency did not significantly differ among wild-type and NHEJ mutant plants. Thus, NHEJ mutants that increase stable transformation frequency do not alter nuclear T-DNA uptake and expression.

We confirmed that cNHEJ genes do not alter transient transformation frequency by infecting leaf disks from VIGS-silenced *N. benthamiana* plants. Figure 3(a) shows the results of these infections using *A. tumefaciens* GV2260(pBISN1). Early after infection (2 or 5 days), silenced and non-silenced plants expressed similar levels of GUS activity. However, later in infection (10, 15, and 25 days) *KU70*-, *KU80*-, and *LIG6*-silenced plants showed more GUS activity than did *LIG4*-silenced plants, or control plants. Early GUS expression likely corresponds to transient transformation, because neither control nor cNHEJ VIGS-silenced plants showed any GUS activity at these early times when infected with an *Agrobacterium* strain harboring the promoter-trap vector pKM1 (Figure 3b). pKM1 contains a promoterless gusA gene near the T-DNA right border (Mysore *et al.*, 1998). Plant tissues infected by this strain express GUS activity only if T-DNA integrates into the plant genome downstream of an active promoter.

### Some NHEJ mutants and silenced plants integrate higher levels of T-DNA into the plant genome

We infected root segments of wild-type and cNHEJ mutant Arabidopsis plants with *A. tumefaciens* GV3101 harboring pKM1. Figure 1(b) shows that *ku80*, *ku70*, and *lig6* mutants had 2- to 3-fold more *GUS* expression than did wild-type, *atm*, *atr*, and *lig4* mutants. These results confirm those of the tumorigenesis assays. We obtained similar results with a promoter-trap assay of *N. benthamiana* plants silenced for cNHEJ genes (Figure 3b). *NbKU70*, *NbKU80* and *NbLIG6* silenced plants showed significantly more GUS activity than did control plants. These results suggest that mutation or silencing of some cNHEJ genes increases T-DNA integration.

We sought direct biochemical evidence to confirm that mutation of some cNHEJ pathway genes results in increased levels of T-DNA integration. We employed a DNA blotting strategy (Mysore *et al.*, 2000b; Crane and Gelvin, 2007; Kim *et al.*, 2007) to quantify the extent of T-DNA integration into plant DNA of wild-type and cNHEJ mutant roots. We infected root segments with *A. tumefaciens* GV3101(pBISN1). Two days after inoculation, we transferred the roots to callus inducing medium (CIM) containing Timentin (GlaxoSmithKline, UK, http://www.gsk.com) (to kill agrobacteria) and continued to grow calli for 1 month in the absence of selection for transgene expression. We subjected total plant DNA from these calli to DNA blot analysis, using the gusA gene harbored by the pBISN1 T-DNA as a hybridization probe. Subsequent rehybridization of the blot with a radiolabeled rRNA gene probe was used to normalize the amount of gusA DNA, integrated into high molecular weight plant DNA, to total plant DNA in each lane. Densitometric scanning of the autoradiograms quantified the amount of T-DNA integrated into plant DNA from each infection. To address the possibility of contaminating T-DNA on the binary vector in our plant DNA preparations, we included undigested pBISN1 DNA on the blot. We did not detect the plasmid DNA hybridization pattern in the plant DNA lanes (Figure 4), suggesting that the observed hybridization signals derived from T-DNA integrated into plant DNA.

Figure 4 shows that, per microgram of plant DNA, *ku70* and *ku80* mutant plants integrated 1.9- and 1.8-fold more T-DNA, respectively, than did wild-type plants. *lig6* mutant plants integrated 1.7-fold more T-DNA than did their respective wild-type controls. A *lig4*/*lig6* double mutant similarly integrated 2.8-fold more T-DNA than did control plants. These findings are consistent with the transformation data, and directly indicate that expression of *KU70*, *KU80* and *LIG6* limits T-DNA integration.

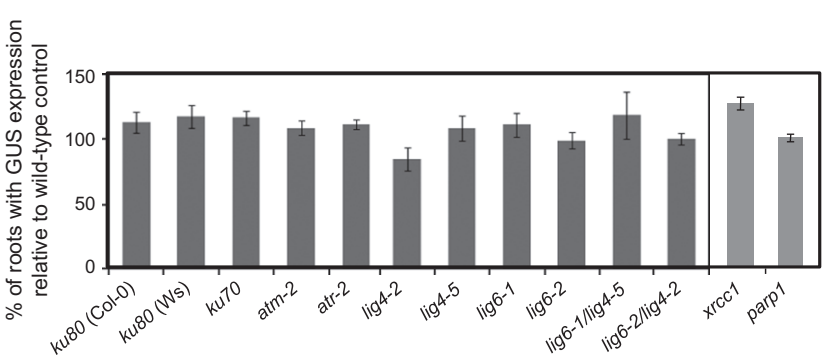


**Figure 2.** Transient transformation assays using Arabidopsis NHEJ mutants.
Root segments from wild-type and NHEJ mutants were inoculated with *A. tumefaciens* GV3101(pBISN1). Bacterial inoculation was at $10^6$ cfu $ml^{-1}$. The roots were stained with X-Gluc 6 days after inoculation.

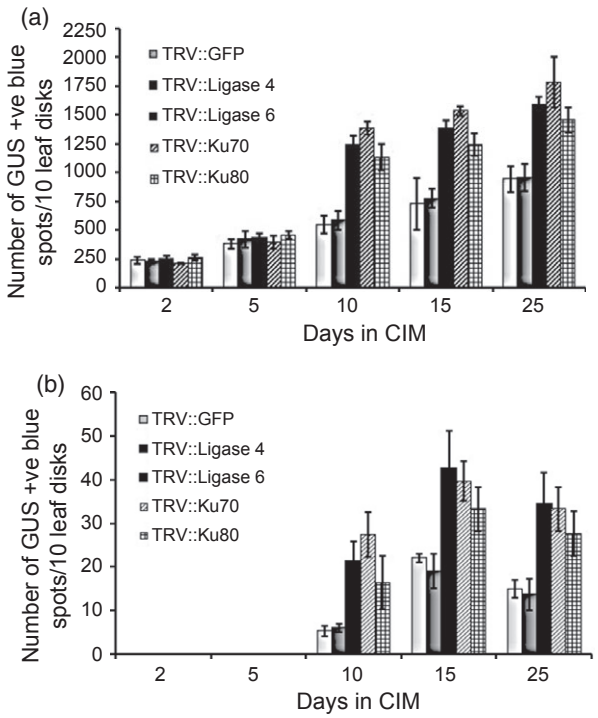


**Figure 3.** Transient transformation assays using VIGS-silenced *N. benthamiana* plants.
(a) Leaf disks of VIGS-silenced *N. benthamiana* plants were infected with *A. tumefaciens* GV2260(pBISN1). Leaf disks were placed on callus inducing medium (CIM) and assayed for GUS activity with X-Gluc. Each bar represents the mean ± standard error (SE) from three replicates (10 disks per replicate).
(b) Leaf disks of VIGS-silenced *N. benthamiana* plants were infected with *A. tumefaciens* GV2260(pKM1). Leaf disks were placed on CIM and assayed for GUS activity with X-Gluc. Each bar represents the mean ± SE from three replicates (10 disks per replicate).

We further investigated the amount of T-DNA integrated into the genome of bNHEJ mutants. The *xrcc1* mutant integrated an amount of T-DNA similar to that of wild-type plants. Interestingly, in three separate infections, the *parp1* mutant integrated 2.6- to 10.4-fold more T-DNA than did wild-type plants (Figure 5a).

We validated the wild-type and *parp1* mutant DNA blot results by qPCR analysis (Figure 5b). Levels of gusA DNA incorporated into total plant DNA were quantified relative to levels of *EF1a* DNA, and T-DNA incorporated into the *parp1* mutant plant DNA was then compared to T-DNA incorporated into wild-type plant DNA. This analysis, conducted on the same DNA as shown in the first *parp1* sample in Figure 5(a), confirmed that the *parp1* mutant integrated more gusA T-DNA than did wild-type plants.

### Simultaneous mutation of genes encoding proteins in each of the NHEJ pathways does not decrease stable transformation or T-DNA integration

Because the cNHEJ and bNHEJ DNA repair pathways may compensate for each other if one of the pathways were disrupted, we generated a homozygous *ku80*/*parp1* double mutant (*ku80:* SAIL_714_A04 × *parp1:* SALK_140400; Figure S6). This double mutant eliminates expression of *KU80*, a key component of the cNHEJ pathway, and *PARP1*, an essential signaling component of the bNHEJ pathway. We tested this mutant, and the corresponding single mutants and wild-type plants, for stable transformation susceptibility and T-DNA integration.

Figure 6 shows that, as shown in Figure 1(a), a *parp1* mutant had approximately the same stable transformation susceptibility as did wild-type plants, whereas a *ku80* mutant had increased transformation susceptibility. The *ku80*/*parp1* double mutant showed transformation susceptibility similar to that of wild-type plants, indicating that simultaneous disruption of genes in both NHEJ pathways had little effect on stable transformation.

We again tested the extent of T-DNA integration into the genomes of wild-type and NHEJ mutant plants in the absence of selection for transgene expression. For each experimental point, we pooled roots of 10 plants; we conducted three replicates for each plant genotype. Figure 7 shows the results of these experiments, conducted using DNA qPCR. Although there was variation among infections, quantitative PCR analysis of total DNA from calli derived from the infected roots showed that, in general, the *ku80* mutant integrated vastly more T-DNA than did wild-type plants. The *parp1* mutant integrated somewhat more DNA, whereas the *ku80*/*parp1* double mutant integrated statistically similar ($P < 0.32$) amounts of T-DNA as did wild-type plants. The greatly increased T-DNA integration in the *ku80* mutant of this experiment, compared with the lesser increase described in Figure 4, reflects differences in transformation events during individual experiments. Regardless, more T-DNA was integrated into total plant DNA of a *ku80* mutant than into DNA of wild-type plants. In addition, *ku80* mutant plants consistently displayed a higher stable transformation frequency than did wild-type plants.

To test whether detection of the gusA gene analyzed above resulted from *Agrobacterium* contamination of the plant DNA samples, we conducted DNA blot analysis of high-molecular-weight plant DNA from the infected roots (Figure S7). The exposure of the blot was such that only the high amounts of integrated T-DNA in the *ku80* genome were detected. These results paralleled those of the qPCR analysis of Figure 7. Most importantly, the hybridization pattern of the gusA probe was similar to that of the ethidium bromide-stained total plant DNA, but different from that of the gusA gene on the T-DNA binary vector pBISN1 used to infect the plants. Controls included DNA from uninfected calli deliberately 'spiked' with *Agrobacterium* harboring pBISN1, and DNA from calli deliberately 'spiked' with isolated pBISN1 DNA. In all controls, the migration of the gusA hybridizing fragment was the same, and different

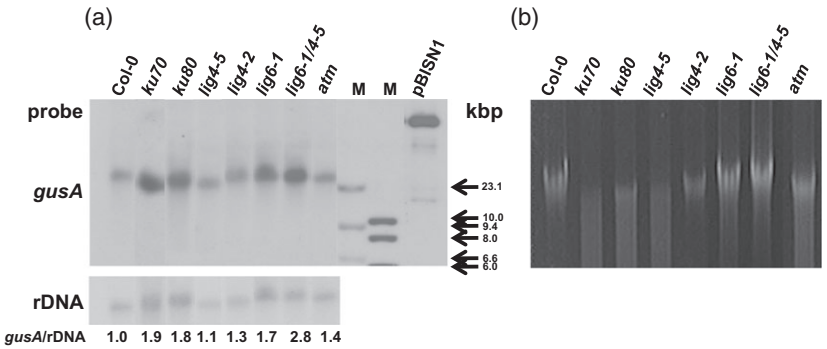


**Figure 4.** DNA blot showing T-DNA integration into high-molecular-weight plant DNA after *Agrobacterium* infection of wild-type and cNHEJ mutants.
Root segments from wild-type and cNHEJ mutant plants were inoculated with $10^8$ cfu $ml^{-1}$ *A. tumefaciens* GV3101(pBISN1). After 2 days, the roots were transferred to CIM containing Timentin and incubated for 4 weeks before DNA extraction.
(a) Blots containing 10 μg of genomic DNA were hybridized with gusA or rDNA probes. Nondigested pBISN1 served to show that plant DNA was not contaminated with *Agrobacterium* DNA. gusA signal strength was normalized to the rDNA probe, and then to infection of wild-type plants (relative intensity). M, DNA size marker.
(b) Ethidium bromide-stained gel, showing the pattern of plant high-molecular-weight DNA, used for the blot in panel (a).

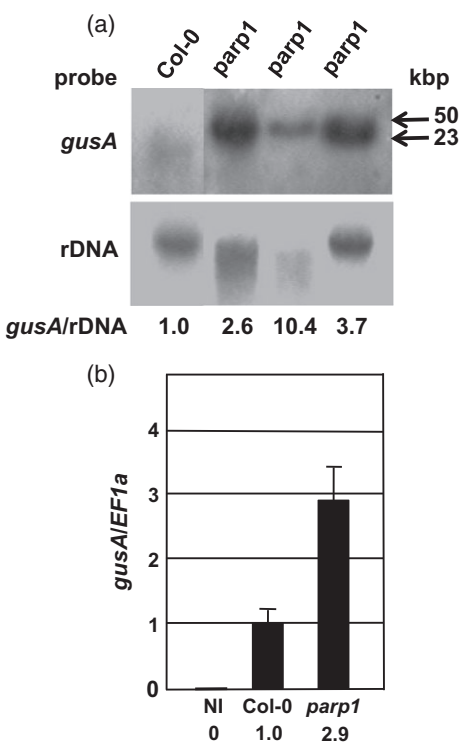


**Figure 5.** A *parp1* mutant integrates more DNA than do wild-type plants.
(a) The amount of gusA DNA incorporated into high molecular weight plant DNA after *Agrobacterium* infection of wild-type and three independent pools of *parp1* mutant root segments. The analysis was conducted as in Figure 4.
(b) The amount of gusA DNA incorporated into high-molecular-weight plant DNA after *Agrobacterium* infection of wild-type and *parp1* mutant root segments by quantitative real-time PCR (qPCR). The relative amount of gusA DNA was normalized to the amount incorporated into wild-type (Col-0) DNA. The DNA sample was the same as that used in the first *parp1* DNA sample shown in panel (a); NI, non-infected. Error bars indicate the standard error (SE) of three technical replicates.

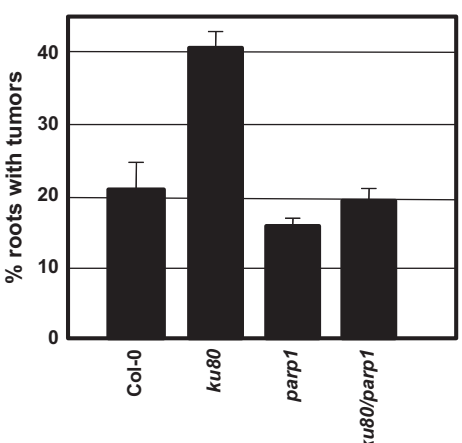


**Figure 6.** Stable transformation of wild-type (Col-0), *ku80*, *parp1*, and *ku80*/*parp1* mutant plants.
Root tumorigenesis assays were conducted as described in the legend to Figure 1(a), using $10^6$ cfu $ml^{-1}$ *A. tumefaciens* A208.

from that of T-DNA in the infected root samples. These results indicate that the gusA gene detected in the infected root samples had integrated into high molecular weight plant DNA, and that the hybridization signal was not the result of agrobacteria contaminating the plant samples.

### T-DNA integrated into the genome of *parp1* mutant plants is more highly methylated than is T-DNA integrated into the genome of wild-type plants

Our finding that *parp1* mutant plants integrate more T-DNA than do wild-type plants while showing the same transformation susceptibility suggests that transgenes integrated into the *parp1* genome may not always be

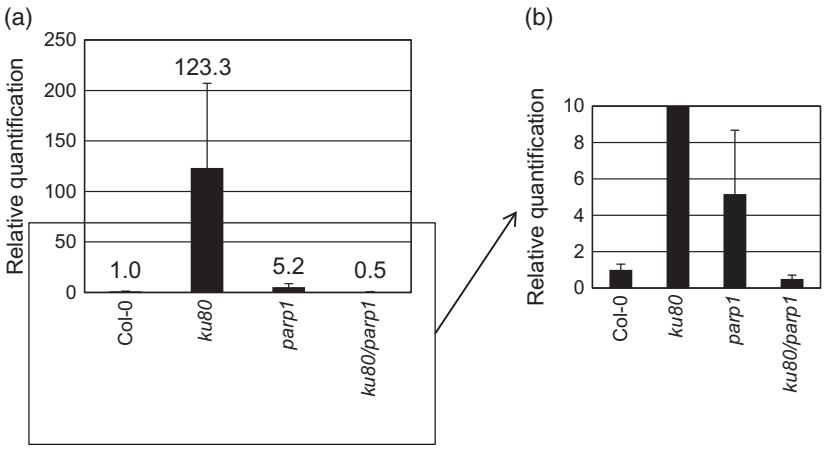


**Figure 7.** Incorporation of T-DNA into plant high-molecular-weight DNA of wild-type, *ku80*, *parp1*, and *ku80*/*parp1* mutant plants.
(a) DNA qPCR analyses of DNA extracted from calli of root segments inoculated with $10^8$ cfu ml$^{-1}$ *A. tumefaciens* At849. Pooled root segments from 10 plants were analyzed for each sample. For each plant genotype, three independent experiments were conducted, and the averages ± standard error (SE) are shown. The analyses quantified the amount of gusA DNA present relative to the amount of an endogenous *EF1α* gene.
(b) Enlargement of the data in panel (a).

expressed. In eukaryotes, DNA methylation is an epigenetic mark that may correlate with gene inactivity. We therefore examined the extent of DNA methylation of the gusA transgene incorporated into high-molecular-weight plant DNA of wild-type and *parp1* mutant plants.

*Msp*I and *Hpa*II are isoschizomeric restriction endonucleases that recognize the sequence 5′-CCGG-3′ but display differential sensitivity to cytosine methylation. Cutting at these sites is blocked for *Msp*I when the external C is methylated, whereas cutting for *Hpa*II is blocked when the internal C is methylated. We attempted to amplify a fragment of gusA from the genomes of transgenic calli by PCR, without or subsequent to digestion of genomic DNA with *Msp*I or *Hpa*II. This amplicon contains three *Msp*I/*Hpa*II sites (Figure 8a) and, if any of these sites are not methylated, amplification cannot occur after restriction endonuclease digestion. If all three sites are methylated, amplification can occur.

Figure 8(b) shows that, without prior digestion by either restriction endonuclease, we could amplify a 738-bp fragment from the DNA of both wild-type and *parp1* mutant transformed cells. As expected, the greater amount of gusA DNA integrated into the *parp1* mutant resulted in a greater production of the gusA amplicon in this mutant. Prior digestion of both samples by *Hpa*II resulted in similar amounts of amplification, although these amounts were less than that of undigested DNA. These results indicate that in both wild-type and *parp1* mutant plants, a similar subset of gusA transgenes was methylated at the internal C residue of all three *Hpa*II sites. However, when the samples were digested with *Msp*I prior to the PCR, we could efficiently amplify a gusA fragment only from the *parp1* sample. These results indicate that the gusA transgene was methylated on the external C residue of all three *Msp*I sites in a subset of the genomes from the *parp1* mutant, but at only barely detectable levels from wild-type plants. Thus, a gusA transgene is more extensively methylated in *parp1* mutant than in wild-type plant DNA.

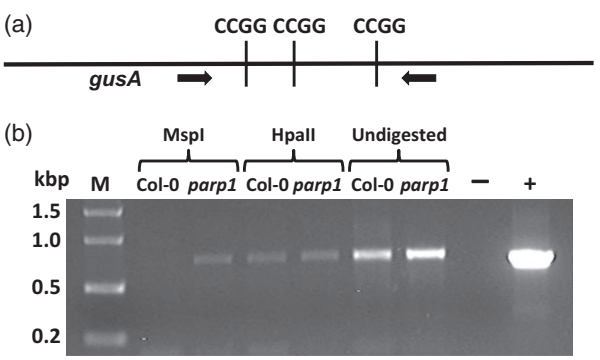


**Figure 8.** PCR analysis of transgene DNA methylation in wild-type and *parp1* mutant root-derived calli.
(a) Schematic diagram of the gusA gene region analyzed. Arrows indicate PCR primers.
(b) Root segments were inoculated with *A. tumefaciens* At849, containing a gusA-intron gene in the T-DNA, at $10^8$ cfu ml$^{-1}$. After 2 days co-cultivation, the bacteria were killed by transfer of the segments to solidified callus inducing medium (CIM) plus Timentin, and calli were grown for 1 month in the absence of selection. After a further 1 week of growth in liquid CIM plus Timentin, DNA was extracted and subjected to PCR prior or subsequent to digestion with *Msp*I or *Hpa*II. The primers amplify a 738-bp fragment of the gusA-intron transgene. M, DNA size standards; –, negative PCR control (water only, no DNA); +, positive PCR control (amplification with a gusA-intron template).

## DISCUSSION

T-DNA integration, followed by transgene expression, are the final steps in stable *Agrobacterium*-mediated plant genetic transformation. The mechanism of integration is poorly understood, and several different models exist to explain this stable transformation process (Tzfira *et al.*, 2004). Integration models have generally relied on examination of sequenced T-DNA/plant DNA junctions, and on elucidation of integrated T-DNA organization. From these observations, scientists derived models to explain what must have occurred during integration. Individual models are compatible with many, but not all, observed integration events. The T-strand invasion model accounts for microhomologies frequently observed between T-DNA and

target site sequences, but does not adequately explain the generation of T-DNA 'head-to-head' and 'tail-to-tail' dimers. Conversely, the dsDNA break repair integration model can account for the formation of these dimers but not the observed microhomologies.

**What is known about T-DNA integration and associated proteins involved in this process?**

Although several reports suggested that T-DNA preferentially integrates into gene-rich regions, promoters, and transcriptionally active DNA (Brunaud *et al.*, 2002; Szabados *et al.*, 2002; Alonso *et al.*, 2003; Chen *et al.*, 2003; Sallaud *et al.*, 2004; Schneeberger *et al.*, 2005; Li *et al.*, 2006) these observations were derived from analyzing integration sites from transgenic plants under selection for transgene expression. Elimination of this 'selection bias' revealed that T-DNA integration appears to be truly random (Kim *et al.*, 2007). Thus, direction of T-DNA to a particular integration site and its integration likely depend on 'general' chromatin proteins, such as histones (Mysore *et al.*, 2000b), CAF-1 (Endo *et al.*, 2006), histone deacetylases (Crane and Gelvin, 2007), and SGA1 (Crane and Gelvin, 2007). Although VirD2 protein may be involved in T-DNA integration (Tinland *et al.*, 1995; Mysore *et al.*, 1998), plant-encoded proteins clearly play the most important role in this process. For example, VirD2 is not responsible for T-DNA ligation to plant chromosomes (Ziemienowicz *et al.*, 2000).

**T-DNA integration and the role of dsDNA break repair proteins**

Genome target sites for T-DNA integration lack extensive homology with T-DNA sequences, and homologous recombination between incoming T-DNA molecules and chromosomal sequences is extremely inefficient (e.g. Hanin *et al.*, 2001). Therefore, homologous recombination is likely not a major pathway for integration. T-DNA molecules preferentially integrate into plant dsDNA break sites (Salomon and Puchta, 1998; Chilton and Que, 2003; Tzfira *et al.*, 2003). Thus, NHEJ has become the preferred model for T-DNA integration. Indeed, in yeast, 'classical' NHEJ proteins are important for T-DNA integration (van Attikum *et al.*, 2001; van Attikum and Hooykaas, 2003). The 'classical' NHEJ pathway generally initiates with binding of the KU70/KU80 heterodimer to broken DNA ends. In mammals the KU/DNA complex attracts the catalytic subunit of DNA-PKcs, which phosphorylates itself and other proteins involved in NHEJ. Artemis (not yet identified in plants) processes DNA prior to ligation by a complex containing LIG4, XRCC4, and XRCC4-like factor (XLF) (Mladenov and Iliakis, 2011). The ATM and ATR kinases are involved in signaling following the generation of dsDNA lesions. We thus initially chose to examine the consequences on *Agrobacterium*-mediated transformation of reducing expression of genes encoding cNHEJ proteins.

Several studies have investigated the role of cNHEJ proteins in *Agrobacterium*-mediated plant transformation. Many have come to opposite conclusions. For example, Gallego *et al.* (2003) and Jia *et al.* (2012) observed no difference in stable transformation efficiency between wild-type and *ku80* mutant plants. However, two reports in Arabidopsis (Friesner and Britt, 2003; Li *et al.*, 2005) and one in rice (Nishizawa-Yokoi *et al.*, 2012) showed that a *ku80* mutant has lower transformation efficiency. The importance of LIG4 in transformation also remains controversial. Friesner and Britt (2003) showed that *lig4* mutants had decreased flower-dip transformation frequency, whereas a study by van Attikum *et al.* (2003) showed no transformation frequency difference between roots of wild-type and mutant plants. These disparate outcomes may result from differences in transformation target tissues. Many Arabidopsis studies were conducted using floral dip transformation assays (van Attikum *et al.*, 2003; Friesner and Britt, 2003; Gallego *et al.*, 2003). However, roots are the natural target for *Agrobacterium*-mediated transformation. Two studies in which roots were infected showed no reduction in transformation frequency using *lig4*, *ku70*, or *ku80* mutants (van Attikum *et al.*, 2003; Jia *et al.*, 2012) and another study indicated that reduced *XRCC4* expression in Arabidopsis and *Nicotiana benthamiana* increased stable transformation and T-DNA integration (Vaghchhipawala *et al.*, 2012). Recently, Mestiri *et al.* (2014) investigated stable *Agrobacterium*-mediated transformation of various Arabidopsis mutants deficient in single or multiple DNA break repair pathways. They observed a small decrease in transformation of single mutants in the cNHEJ pathway (*ku80*), the bNHEJ pathway (*xrcc1* and *xpf*), in homologous recombination (*xrcc2*), and in lines with multiple mutations (*ku80*/*xrcc1* and *ku80*/*xrcc1*/*xpf*). However, they showed a greater decrease in transformation in the *ku80*/*xrcc1*/*xpf*/*xrcc2* quadruple mutant. It is not clear why their c- and bNHEJ mutants showed a decrease in transformation susceptibility and ours did not. However, the decrease shown by Mestiri *et al.* (2014) was small (2- to 3-fold), and differences in experimental technique and/or experimental materials between the groups may have caused the different observations. We note that our mutant Arabidopsis lines did not show altered growth or developmental characteristics, and that leaf tissue from our VIGS-silenced *N. benthamiana* lines callused at a rate similar to that of tissue from wild-type plants (Figure S5).

In this study, we examined the effect on stable transformation of inhibiting expression of both 'classical' and 'backup' NHEJ proteins, either by the use of Arabidopsis mutants or by VIGS analysis of *N. benthamiana.* The results of these studies were both consistent and surprising. Decreasing or abolishing NHEJ gene expression had either little effect on (*lig4*, *atm*, *atr*, *xrcc1*, and *parp1*), or increased stable transformation (*ku70*, *ku80*, and *lig6*). One

of the Arabidopsis *ku80* mutants we investigated was identical to that used by Li *et al.* (2005). Our results indicated transformation hyper-susceptibility of this mutant; Li *et al.* described this mutant as having decreased susceptibility. We note that Li *et al.* (2005) used only a 'high' (unspecified in their manuscript) *Agrobacterium* inoculum concentration. We used inoculum concentrations ranging from $10^5$ to $10^8$ cfu $ml^{-1}$, with consistent results. Our results are also consistent with those of Jia *et al.* (2012) who showed that an Arabidopsis *ku80* mutant is not recalcitrant to *Agrobacterium*-mediated root transformation. Jia *et al.* (2012) used a high *Agrobacterium* inoculation concentration, therefore they would not have been able to detect the hyper-susceptibility that was seen by our group.

Our stable transformation results indicate that 'classical' and 'backup' NHEJ proteins do not positively contribute to transformation susceptibility. Indeed, our data indicate that the presence of these proteins may limit stable transformation. Additionally, there was no effect of inhibiting expression of NHEJ proteins on transient transformation, a process that does not require T-DNA integration. Most importantly, biochemical analysis of Arabidopsis *ku70*, *ku80 lig6*, and *parp1* mutants indicated an increase in T-DNA integration into high-molecular-weight plant DNA. Vaghchhipawala *et al.* (2012) explained transformation hyper-susceptibility of a *XRCC4* down-regulated Arabidopsis line as a consequence of slower joining of either naturally occurring dsDNA breaks, or breaks induced by the stress of incubating plant cells with agrobacteria; the data presented in this paper are consistent with this interpretation. Slower break processing and sealing would provide increased opportunity for T-DNA ligation to the broken ends of plant DNA and mirrors the enhancement of transformation upon induction of DNA damage in protoplasts (Köhler *et al.*, 1989). Evidence for the accumulation of DNA breaks in the NHEJ mutant lines is provided by the constitutive activation of the DNA damage response, increased DNA fragmentation, increased yellowing and root growth inhibition (Figure S2), and the higher incidence of cell death in these lines (Gallego *et al.*, 2003; Jia *et al.*, 2012; Vaghchhipawala *et al.*, 2012).

The greatly increased quantity of T-DNA integrated into high-molecular-weight DNA of the *parp1* mutant, in the absence of an increase in transformation susceptibility, was a surprising result. On the one hand, this result indicates that PARP1 activity limits T-DNA integration, as do the activities of KU70, KU80, LIG6 (this study), and XRCC4 (32). PARP1 activity is important for preventing double-stranded DNA breaks (Huber *et al.*, 2004; Woodhouse *et al.*, 2008; Jia *et al.*, 2013) that may provide sites for T-DNA integration, for recruiting XRCC1 to double-stranded DNA break sites to repair these breaks (Masson *et al.*, 1998), and for suppressing homologous recombination (Puchta *et al.*, 1995). On the other hand, our results suggest that PARP1 activity may increase transgene expression, or inhibit transgene silencing. PARP1 can poly(ADP-ribosyl)ate chromatin proteins, including HON1, that may affect chromatin structure (Poirier *et al.*, 1982). By interacting with and modifying the DNA methyltransferase DNMT1 in animal cells (Althaus, 2005; Reale *et al.*, 2005; Zampieri *et al.*, 2012), PARPs can suppress DNA methylation and increase transgene expression (Caiafa *et al.*, 2009; Ciccarone *et al.*, 2012). Indeed, our analysis of the methylation status of a gusA transgene integrated into plant DNA indicates that the transgene is more heavily methylated at CXG residues (a target site for the plant DNA methyltransferases CMT3 and DRM2/3; Law and Jacobsen, 2010) in *parp1* mutant plants than in wild-type plants. Zardo *et al.* (1999) showed that a plasmid harboring a chloramphenicol acetyltransferase (*CAT*) reporter gene remained unmethylated and active when introduced into mouse fibroblast cells. However, if the cells were pretreated with the PARP inhibitor 3-aminobenzamide (3-ABA), the plasmid DNA became methylated and CAT activity decreased. Our results, showing that increased transgene copy number does not result in a concomitant increase in transformation frequency of a *parp1* mutant, and that transgene DNA is more heavily methylated in a *parp1* mutant, similarly suggest that PARP activity in Arabidopsis is required for full transgene expression activity.

**An alternate NHEJ pathway is used in animal cells for a number of purposes**

An alternate 'backup' NHEJ pathway exists in animal cells that is important for several biological processes, including repair of radiation-induced dsDNA breaks, immunoglobin gene class switch recombination, formation of chromosome translocations, and telomere maintenance. This pathway uses PARP1 and HON1 rather than the KU protein complex to align dsDNA breaks, the MRN complex rather than Artemis to process DNA at break sites, and XRCC1 and DNA ligase III (LIG3) rather than XRCC4 and LIG4 to seal breaks (Mladenov and Iliakis, 2011). Plants do not contain DNA LIG3, but rather encode LIG6 in addition to DNA ligases I (LIG1) and LIG4 (Waterworth *et al.*, 2009, 2010). DNA LIG1 is important for plant development and repair of both single-stranded and double-stranded DNA breaks (Waterworth *et al.*, 2009). The only known role of LIG6 is in seed viability and low temperature stress upon long-term storage (Waterworth *et al.*, 2010). Our current data, and those of others (van Attikum *et al.*, 2003), indicate that LIG4 is not essential for *Agrobacterium*-mediated root transformation. In addition, the plant-specific LIG6 appears to limit transformation. These results suggest that another DNA ligase activity, perhaps LIG1, is responsible for joining T-DNA to plant DNA. This hypothesis is supported by recent data indicating that LIG1 is involved in a highly active pathway of dsDNA break repair in Arabidopsis (Waterworth *et al.*,

2009). Our results are consistent with the hypothesis that the KU70/80 complex limits T-DNA integration (Vaghchhipawala *et al.*, 2012). The alternate mammalian NHEJ pathway uses HON1, rather than the KU complex, for DNA break strand alignment. Interestingly, RNAi-mediated silencing of a histone H1-like protein resulted in decreased Arabidopsis transformation (Crane and Gelvin, 2007).

### What mechanism does T-DNA use for integration?

Because T-DNA integration does not use homologous recombination, nor are the 'classical' or 'backup' NHEJ pathways required, what pathway does it use? *In vitro* end joining of dsDNA breaks using extracts from KU- or LIG4-deficient animal cells indicated increased frequency of microhomology use during repair processes (Feldmann *et al.*, 2000). In addition, KU80-, LIG4-, and XRCC4-deficient animal cells show increased chromosome translocations (Boboila *et al.*, 2010). The use of DNA microhomologies during T-DNA integration and the formation of chromosome translocations are frequent hallmarks of *Agrobacterium*-mediated transformation (Mayerhofer *et al.*, 1991; Clark and Krysan, 2010). These observations suggest that T-DNA may sometimes use the bNHEJ pathway for integration. Our initial findings that disruption of the cNHEJ or the bNHEJ pathway does not decrease *Agrobacterium*-mediated stable transformation or T-DNA integration suggest that these two pathways may be redundant with regard to T-DNA integration. However, simultaneous disruption of *ku80* and *parp1* also does not decrease transformation relative to the frequency of transformation of wild-type (Col-0) plants. Although our results are quantitatively different from those of Mestiri *et al.* (2014), both groups report that simultaneous disruption of multiple DNA break repair pathways does not eliminate transformation, suggesting that there is some as yet unknown pathway that mediates T-DNA integration.

## EXPERIMENTAL PROCEDURES

### Bacterial and plant growth

*A. tumefaciens* was cultured in liquid AB-sucrose or LB media at 28°C with the appropriate antibiotics (rifampicin, 10 mg $L^{-1}$; kanamycin, 25 mg $L^{-1}$; spectinomycin, 50 mg $L^{-1}$; Zhu *et al.*, 2003). *Arabidopsis thaliana* seeds were surface sterilized and germinated at 25°C in Petri dishes on solidified Gamborg's B5 medium (Caisson Laboratories, Logan, UT, USA, http://www.caissonlabs.com) containing 100 mg $L^{-1}$ Timentin. Individual plants were moved to baby food jars containing solidified B5 medium and grown for 3 weeks under long-day (14 h light) conditions at 25°C. Plants were collected before flowering for transformation assays as described (Nam *et al.*, 1999; Mysore *et al.*, 2000a; Zhu *et al.*, 2003). For bleomycin sensitivity assay, sterilized seeds were germinated on Petri plates with MS medium solidified with 0.2% Phytagel™ (Sigma-Aldrich, USA, http://www.sigmaaldrich.com). Bleomycin sensitivity assay was done as described earlier (Vaghchhipawala *et al.*, 2012). The Arabidopsis genes and ecotypes investigated, and the corresponding mutants, are: *KU70* [At1g16970; Col-0: SALK_123114 (Heacock *et al.*, 2007; Kannan *et al.*, 2008; Qi *et al.*, 2013)]; *KU80* (At1g48050; Col-0: SAIL_714_A04; Ws: FLAG_396B06); *ATM* [At3g48190; Col-0: SALK_006953 (Vespa *et al.*, 2005)]; *ATR* [At5g40820; Col-0: SALK_032841 (Vespa *et al.*, 2005)]; *LIG4* [At5g57160; Col-0: SALK_044027 (*Atlig4-2*); Col-0: SAIL_597_D10 (*Atlig4-5*, Waterworth *et al.*, 2010)]; *LIG6* [At1g66730; Col-0: SALK_079499-1 (*Atlig6-1*, Waterworth *et al.*, 2010); Ws: FLAG_437H07 (*Atlig6-2*, Waterworth *et al.*, 2010)]; *XRCC1* (At1g80420; Col-0: SALK_125373); *PARP1* [At4g02390; Col-0: SALK_140400 (Boltz *et al.*, 2014)]. *N. benthamiana* seeds were germinated in Metromix 830 (SunGro, AR, USA, http://www.sungro.com). Plants in 10 cm diameter pots were fertilized (20-10-20), given a soluble trace element mix (The Scotts Co., Marysville, OH, USA), and maintained at 23°C, 70% humidity, 16 h light/8 h dark photoperiod. Three-week-old plants were used for VIGS experiments.

### Transient and stable Arabidopsis root transformation assays

For transient transformation assays, root segments from five plants were pooled and infected with *A. tumefaciens* GV3101 containing pBISN1 (Narasimhulu *et al.*, 1996) as described (Zhu *et al.*, 2003). For the promoter-trap assay, *A. tumefaciens* GV3101 containing pKM1 (Mysore *et al.*, 1998) was used. After co-cultivation on hormone-free MS medium for 48 h, roots were transferred to CIM containing 100 mg $L^{-1}$ Timentin for 2 days (or 3, 7, and 13 days for the promoter-trap assay), then stained with X-Gluc overnight at 37°C. Root segments were washed with 70% ethanol and blue-stained segments counted using a stereomicroscope. For stable transformation assays, root segments pooled from five plants were inoculated with the tumorigenic strain *A. tumefaciens* A208. After 2 days, the segments were separated on plates containing MS medium plus 100 mg $L^{-1}$ Timentin and incubated at 24°C for 3–4 weeks, then scored for tumor development. For both transformation assays, 3–5 pools of root segments were inoculated at various bacterial concentrations ($10^5$–$10^8$ cfu $ml^{-1}$) and the results reported as percent positive ± standard error.

### Tobacco rattle virus vectors and plasmids

VIGS vectors pTRV1 and Gateway®-enabled pTRV2 were obtained from Dr S.P. Dinesh-Kumar. *N. benthamiana* homologues of Arabidopsis NHEJ pathway genes were identified by BLASTX from Solanaceae Genomics Network and JCVI databases. *N. benthamiana* cDNAs were amplified by RT-PCR using gene-specific primers (Table S1) and cloned into pGEM-T (Promega Corp., Madison, WI, USA, http://www.promega.com). Clones for each of the candidate genes were sequenced to confirm their identity. Using pGEM-T clones as template, the target genes were PCR amplified with specific primers containing adapter (*attB1* 5′- GGGGACAAGTTTG TACAAAAAAGCAGGCT-3′ and *attB2* 5′-GGGGACCACTTTGTACAA GAAAGCTGGGT-3′) sequences in the forward and reverse primers, respectively (Table S2). PCR products were cloned into the VIGS vector pTRV2 (Anand *et al.*, 2007) by Gateway® cloning following the manufacturer's guidelines (Invitrogen Life Technologies, Carlsbad, CA, USA). pTRV2 derivatives were introduced into *A. tumefaciens* GV2260.

### Virus infection of *N. benthamiana* by *Agrobacterium*-mediated infiltration

Agroinoculations for VIGS were performed using the leaf infiltration method as described (Anand *et al.*, 2007). Ten plants were

inoculated for each clone and the experiments were repeated twice.

### Leaf disk transformation assays of *N. benthamiana*

Leaves from silenced and TRV::*GFP* inoculated control plants were collected 3 weeks post-silencing and assayed as described (Anand *et al.*, 2007). For tumorigenesis assays, leaf disks were infected with *A. tumefaciens* A348. For callus assays, leaf disks were infected with *A. tumefaciens* GV2260(pCAS1) (Nam *et al.*, 1999) and were transferred to CIM plus 5 mg $L^{-1}$ ppt, 250 mg $L^{-1}$ cefotaxime, and 100 mg $L^{-1}$ ticarcillin and incubated at 25°C. After 4 weeks, the number of leaf disks with ppt-resistant calli was scored. For transient transformation assays, leaf disks were inoculated with *A. tumefaciens* GV2260 containing pBISN1 and were transferred onto CIM containing 250 mg $L^{-1}$ cefotaxime and 100 mg $L^{-1}$ ticarcillin. GUS activity was assayed as described (Mysore *et al.*, 1998). The number of GUS positive blue spots was counted visually using a stereomicroscope. For *N. benthamiana* promoter-trap assays, leaf disks were inoculated with *A. tumefaciens* GV2260 containing pKM1 and incubated for 2 days at 25°C. Leaf disks were transferred onto CIM containing 250 mg $L^{-1}$ cefotaxime and 100 mg $L^{-1}$ ticarcillin. Explants were collected at 2, 5, 10, 15 and 25 days and stained with X-Gluc. The number of GUS positive blue spots was counted using a stereomicroscope.

### RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from leaves of TRV::*GFP* infected *N. benthamiana* target gene silenced plants, Arabidopsis NHEJ mutants, and wild-type plants using a RNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA, USA, http://www.qiagen.com). For each gene silenced in *N. benthamiana*, 15 leaf discs (0.5 cm diameter) from five independent plants (three disks per plant) were used for RNA extraction. Leaf disks were harvested from plants 3 weeks post VIGS. For Arabidopsis, leaves from 4-week-old plants were used. 2.5 μg of total RNA was reverse transcribed using 100 units of SuperScript II RNase H-reverse transcriptase and 500 ng of oligo(dT) 18–24 primer (Invitrogen, Carlsbad, CA, USA). Amplification of specific genes used 300 nM of gene-specific primer (Table S3) and 2 units of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA, USA). Parallel reactions were run with *NbActin* or *AtActin* or *AtEF1-α* primers as controls.

### DNA blot assay

After infection with *A. tumefaciens* GV3101(pBISN1) at either $10^7$ or $10^8$ cfu $ml^{-1}$, Arabidopsis root segments were incubated for 4 weeks on CIM containing 100 mg $L^{-1}$ Timentin. DNA was extracted from the resulting calli using a cetyltrimethylammonium bromide procedure (Weigel and Glazebrook, 2002) and the DNA concentration measured using a NanoDrop apparatus (Thermo Scientific, Waltham, MA, USA, http://www.thermoscientific.com). Ten microgram of total DNA was subjected to electrophoresis through 0.7% agarose gels and the DNA transferred to a Hybond-$N^+$ membrane (Amersham). The gusA gene was amplified from pBISN1 and labeled with $^{32}$P-dCTP using Ready-to-go DNA labeling beads (Amersham). Membranes were prehybridized with hybridization buffer (0.25 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS) plus 100 mg $L^{-1}$ salmon sperm DNA at 65°C overnight. The labeled probe was incubated with the membrane for 12 h. After washing with 2× SSC, 2% SDS at 65°C, hybridization was detected using X-ray film and a phosphorimager. To normalize for differences in DNA loading in the lanes, the blots were re-hybridized with a flax rDNA probe. The X-ray films were scanned using a Umax PowerLook 1100 scanner and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA, http://www.nih.gov).

### Quantitative real-time PCR assay

The amount of gusA DNA integrated into high-molecular-weight plant DNA of the *parp1* single or *ku80*/*parp1* double mutants and wild-type root calli was measured by quantitative real-time PCR (qPCR) using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA, http://www.bio-rad.com). Primer sequences used to amplify the gusA and *EF1α* genes are as follows: gusA, forward, 5′-ATGAAGATGCGGACTTACGTGGCA-3′; gusA reverse, 5′-ATCTGCCCAGTCGAGCATCTCTTC-3′; *EF1α* forward, 5′-TTCACCCTTGGTGTCAAGCA-3′; *EF1α* reverse, 5′-TTTCAT CGTACCTGGCCTTGCA-3′. qPCR reactions were assembled using the SsoFast EvaGreen Supermix (Bio-Rad). The program used for the PCR included an initial temperature of 95°C for 2 min followed by 35 cycles of 95°C for 20 sec, 60°C for 20 sec, and 72°C for 20 sec. The assay was performed in triplicate for each sample to assess technical variability. Data analyses were performed with the Bio-Rad CFX Manager 2.0 Software. The comparative cycle threshold method ($\Delta\Delta C_t$) was used to obtain the relative fold change using *EF1α* as a reference gene.

### DNA methylation assay

Analyses were conducted on DNA from calli derived from roots of wild-type or *parp1* mutant plants infected by *A. tumefaciens* At849. DNA samples (approximately 100 ng) were either untreated or treated with 20 units of *Msp*I or *Hpa*II for 2 h at 37°C, followed by addition of 20 more units of enzyme and incubation for another 2 h. Twenty percent of each sample was subjected to PCR using primers that amplify a fragment of the gusA transgene (5′-ACGATCAGTTCGCCATGC-3′ and 5′-TCCCGCTAGTGCCTTGTCC-3′). Amplification used Phusion High-Fidelity DNA polymerase (Thermo Scientific), with the following conditions: 98°C, 30 sec; followed by 30 cycles of 98°C, 10 sec/60°C, 15 sec/72°C, 15 sec; then extension at 72°C, 5 min. Following amplification, samples were analyzed by electrophoresis through a 1.0% agarose gel.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** RT-PCR analysis of Arabidopsis NHEJ mutants.

**Figure S2.** Bleomycin sensitivity of Arabidopsis cNHEJ mutants.

**Figure S3.** Stable transformation assays using Arabidopsis NHEJ mutants and VIGS-silenced *N. benthamiana*.

**Figure S4.** Virus-induced gene silencing (VIGS) of NHEJ pathway genes in *Nicotiana benthamiana*.

**Figure S5.** Effect of gene silencing on cell division potential.

**Figure S6.** PCR genotyping of the *ku80*/*parp1* double mutant.

**Figure S7.** Integration of T-DNA into the genomes of wild-type (Col-0) and NHEJ mutant plants.

**Table S1.** Primers used for amplification of NHEJ homologs from *Nicotiana benthamiana*.

**Table S2.** Primers used for gateway cloning of *Nicotiana benthamiana* NHEJ cDNA homologs into VIGS vector pTRV2.

**Table S3.** Oligonucleotides used in RT-PCR reactions to demonstrate reduction in transcript levels for genes silenced by VIGS.

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