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Deficiency of both classical and alternative end-joining pathways leads to a synergistic defect in double-strand break repair but not to an increase in homology-dependent gene targeting in Arabidopsis

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SUMMARY

In eukaryotes, double-strand breaks (DSBs) are either repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ). In somatic plant cells, HR is very inefficient. Therefore, the vast majority of DSBs are repaired by two different pathways of NHEJ. The classical (cNHEJ) pathway depends on the heterodimer KU70/KU80, while polymerase theta (POLQ) is central to the alternative (aNHEJ) pathway. Surprisingly, Arabidopsis plants are viable, even when both pathways are impaired. However, they exhibit severe growth retardation and reduced fertility. Analysis of mitotic anaphases indicates that the double mutant is characterized by a dramatic increase in chromosome fragmentation due to defective DSB repair. In contrast to the single mutants, the double mutant was found to be highly sensitive to the DSB-inducing genotoxin bleomycin. Thus, both pathways can complement for each other efficiently in DSB repair. We speculated that in the absence of both NHEJ pathways, HR might be enhanced. This would be especially attractive for gene targeting (GT) in which predefined changes are introduced using a homologous template. Unexpectedly, the *polq* single mutant as well as the double mutant showed significantly lower GT frequencies in comparison to wildtype plants. Accordingly, we were able to show that elimination of both NHEJ pathways does not pose an attractive approach for *Agrobacterium*-mediated GT. However, our results clearly indicate that a loss of cNHEJ leads to an increase in GT frequency, which is especially drastic and attractive for practical applications, in which the *in planta* GT strategy is used.

Keywords: genome engineering/editing, DNA repair and processing, DNA replication, DNA recombination.

INTRODUCTION

DNA is constantly exposed to exogenous and endogenous factors that induce various kinds of damage and, thus, threaten genome integrity in all organisms. Double-strand breaks (DSBs) represent the most cytotoxic DNA lesion. Natural DSBs might arise from ionizing radiation, various chemical agents, oxidative damage, or blocked replication forks (Britt, 1996). Moreover, understanding the mechanisms of DSB repair is important for successfully performing genome engineering experiments using the CRISPR/Cas technology (Beying et al., 2021). Two different main mechanisms exist for repairing DSBs, homologous recombination (HR) and non-homologous end-joining (NHEJ), which can both be further subdivided into subpathways. HR uses homologous sequences as repair templates, resulting in faithful restoration of the original genetic information, while NHEJ is often error-prone and associated with mutations, deletions, or insertions. NHEJ can be subdivided into classical NHEJ (cNHEJ) and alternative NHEJ (aNHEJ). The latter is also referred to as microhomology-mediated end-joining. Characteristic of cNHEJ is that DSB ends are more or less directly joined. The KU70/KU80 heterodimer is essential for this process. It binds free double-stranded DNA ends and protects them from nucleolytic degradation (Mimori & Hardin, 1986). In addition, it is also required to recruit further repair factors (Jones et al., 2001; Mahaney et al., 2009; Uematsu et al., 2007; Walker et al., 2001). Mutation of the KU heterodimer leads to severe growth deficiencies as well as a more rapid aging process in mice (Nussenzweig et al., 1996; Vogel et al., 1999). In contrast, Arabidopsis mutants exhibit a normal phenotype but are hypersensitive to DSB-inducing genotoxic agents (Bundock et al., 2002). Multiple studies

demonstrated that the KU heterodimer plays an important role in DSB repair in plants (Charbonnel et al., 2010; Friesner & Britt, 2003; Tamura et al., 2002; West et al., 2002). Recently, it could be shown that KU70 is also important during DNA repair to keep the correct ends linked together in the presence of more than one DSB to avoid chromosomal rearrangements, especially inversions and translocations (Beying et al., 2020; Schmidt et al., 2019).

In mammalian cells, repair by cNHEJ occurs significantly more frequently than aNHEJ. This is partly due to the fact that the KU heterodimer binds DSB ends with a higher affinity than the initial binding protein poly(ADP-ribose) polymerase 1 (PARP1) in the aNHEJ pathway (Mao et al., 2008). aNHEJ uses short microhomologies of 2–20 bp in the vicinity of the DSB for the repair by POLQ (Deriano & Roth, 2013; Kent et al., 2015; Schimmel et al., 2017). During aNHEJ, POLQ is recruited to the resected DSB ends and mediates the annealing of the short homologous regions, as well as subsequent DNA synthesis to fill the remaining gaps (Ahrabi et al., 2016; Anand et al., 2016; Black et al., 2016; Truong et al., 2013; Zahn et al., 2015). This POLQ-dependent repair pathway is highly mutagenic and results in characteristic repair patterns, such as deletions or insertions of short sequence segments (templated insertions) along the break ends (Black et al., 2016; Roerink et al., 2014).

POLQ is a unique multifunctional protein with a N-terminal helicase and a C-terminal polymerase domain. The two domains are separated by a large central domain (Harris et al., 1996; Seki et al., 2003). POLQ is highly conserved between different organisms, but no orthologous genes have been found in yeast or other fungi (Yousefzadeh & Wood, 2013). In Arabidopsis, a POLQ homolog has been identified but few reports of the detailed functions of the protein in plants have been published so far. In Arabidopsis, the corresponding gene was initially named *TEBICHI* (hereafter referred to as At*POLQ*). Although, at this time, it was not clear that POLQ is involved in NHEJ in any eukaryote, a pioneering study demonstrated that the protein is influencing cell division and differentiation and that its loss leads to an activated DNA damage response in Arabidopsis (Inagaki et al., 2006). Further analysis by the same authors revealed that double mutants in factors involved in HR showed severe growth defects, indicating that AtPOLQ acts in a parallel pathway to HR to safeguard cell proliferation (Inagaki et al., 2009). Just recently, studies have indicated that POLQ is mandatory in avoiding accumulation of DNA damage during DNA replication (Hacker et al., 2022; Nisa et al., 2021). Additionally, POLQ is essential for T-DNA integration in Arabidopsis. It has been postulated that POLQ enables capture of the single-stranded 3′-ends of T-DNA after end resection and thus, contributes to T-DNA integration (Kralemann et al., 2022; van Kregten et al., 2016). However, a recently published study suggested that stable integration of T-DNA is still possible in POLQ-deficient rice, albeit at low frequencies: thus, other minor pathways may exist to stably integrate T-DNA (Nishizawa-Yokoi et al., 2021). While there is not much known about the function of POLQ in plants, there are already a couple of functions characterized in other eukaryotes. However, the functions between different POLQ orthologs vary largely, possibly due to the large variance of the central domain (Yousefzadeh & Wood, 2013). For instance, in POLQ-deficient mammalian cells, high HR rates have been detected, whereas analyses in Arabidopsis indicate the opposite (Inagaki et al., 2006; Mateos-Gomez et al., 2015; van Tol et al., 2022). Nonetheless, POLQ and its orthologs all seem to be implicated in aNHEJ (Chan et al., 2010; Mateos-Gomez et al., 2015; Roerink et al., 2014; Thyme & Schier, 2016; van Kregten et al., 2016). In addition to its role in aNHEJ, POLQ has been described to have a translesion synthesis polymerase activity. This damage-tolerant but often error-prone mechanism can bypass various DNA damages, such as genotoxin-induced crosslinks, and continue replication (Seki et al., 2003, 2004). In contrast to KU heterodimer loss of POLQ in mice does not lead to developmental defects (Leonhardt et al., 1993; Shima et al., 2003).

For mutagenesis, DSB-induced NHEJ represents an attractive pathway that is widely used for gene editing. However, gene targeting (GT), the introduction of predefined modifications by the use of a DNA template, is dependent on HR. Like in mammals, NHEJ represents the dominant mode for DSB repair in plants (Puchta, 2005). Therefore, HR frequencies are low and so are GT efficiencies. Several strategies have been employed to overcome this bottleneck. These approaches mainly focused on the use of improved tools to optimize DSB induction, modification of the donor template and its deployment in close proximity to the DSB, or HR timing in dependence on the cell cycle (Huang & Puchta, 2019). In addition, manipulating the DSB repair machinery poses a promising strategy to increase HR rates. GT could be improved by overexpression of HR-related proteins, such as the yeast protein RAD54 (Shaked et al., 2005), and by suppression of NHEJ-related proteins (Qi et al., 2013). On the other hand, NHEJ-based approaches focused on blocking either cNHEJ (Nishizawa-Yokoi et al., 2012; Qi et al., 2013) or aNHEJ (van Tol et al., 2022). In this study, we wanted to investigate whether GT efficiencies can be increased by blocking both NHEJ pathways simultaneously.

RESULTS

Simultaneous knockout of cNHEJ and aNHEJ results in drastic growth defects

As each of the two NHEJ pathways is important for genome stability in plants, we were interested to test what the consequence of the simultaneous loss of both pathways would be for Arabidopsis. To establish the double

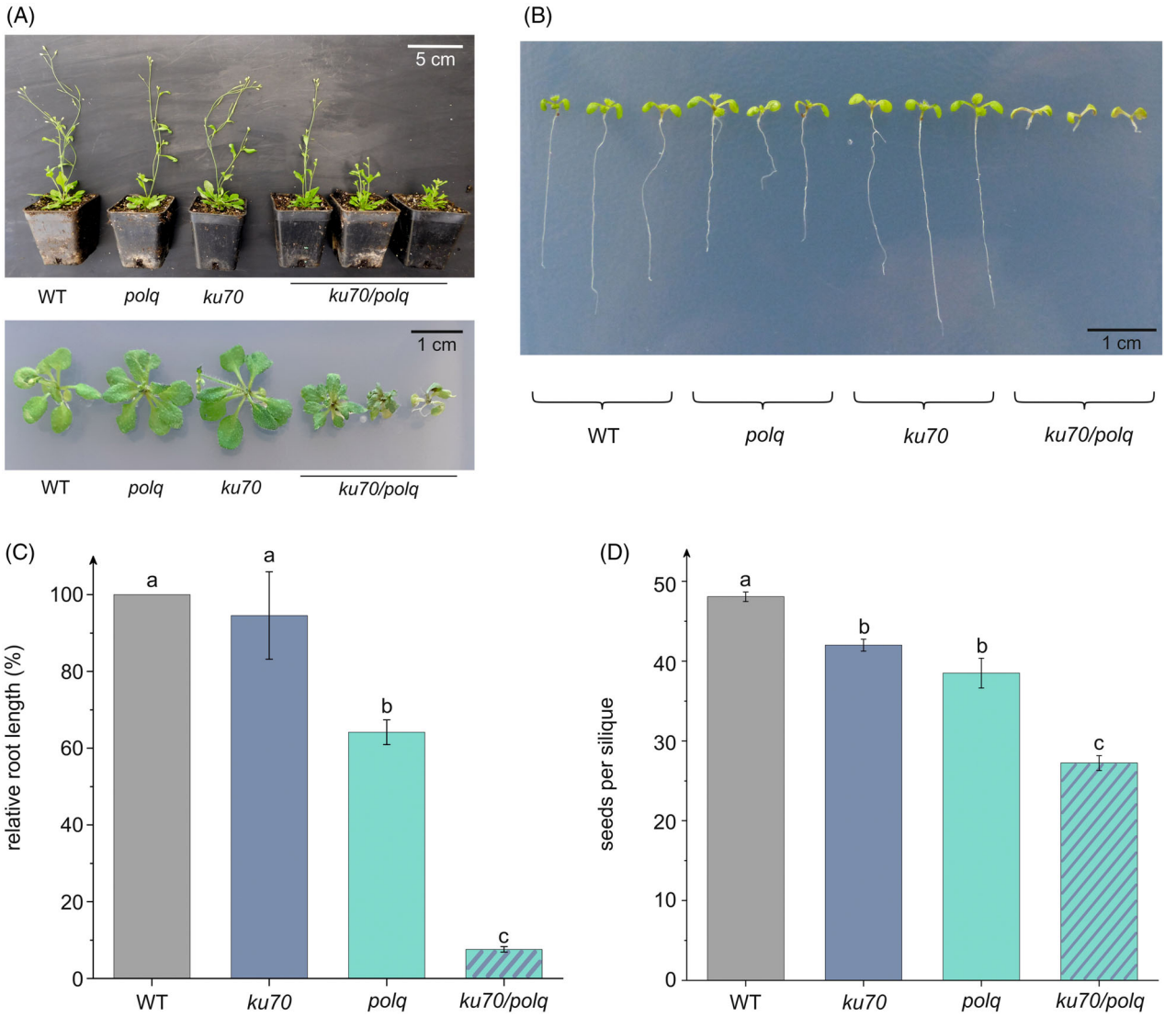


Figure 1. Growth phenotype and fertility analyses of non-homologous end-joining (NHEJ) mutants compared to wildtype.
(A) Shown are 5-week-old (upper figure) plants and 3-week-old (lower figure) seedlings of *ku70/polq* double mutants compared with wildtype (WT) plants and the respective single mutants, all in the Columbia 0 background. The double mutants display strongly impaired growth (upper scale bar = 5 cm, lower scale bar = 1 cm).
(B, C) Root length of 10-day-old seedlings in the *ku70/polq* double mutant is significantly reduced compared to wildtype plants and corresponding single mutants (scale bar = 1 cm, $n = 3$).
(D) Simultaneous loss of AtPOLQ and AtKU70 leads to a significantly reduced amount of seeds per silique in *ku70/polq* double mutant, in contrast to both single mutants and wildtype ($n = 5$). The statistical significance was determined via Fisher LSD test (P-value <0.01), with different lowercase letters representing significant differences.

mutant, two well-characterized homozygous T-DNA single mutants were crossed. The resulting *ku70/polq* double mutant line exhibited severe growth deficiencies (Figure 1A). These were characterized by a small growth phenotype as well as deformed leaves, whereas both single mutants showed no major differences in growth compared to the wildtype. For a more detailed phenotypic analysis, root length was determined (Figure 1B). Thus, it is possible to get a more quantitative estimation on the importance of the respective factors for the repair of replication-associated DNA damage. Since the root meristem is a rapidly dividing tissue, DNA damage that occurs spontaneously during replication may directly affect cell division and thus, hamper root growth (Curtis & Hays, 2007). For the assay, the root length of 10-day-old plants was determined using the program ImageJ with the plugin SmartRoot (Lobet et al., 2011). We confirmed a significantly shortened root length (by almost 36%) of the AtPOLQ-deficient single mutant under our growth conditions compared to wildtype plants (Inagaki et al., 2006).

Interestingly, different studies have reported different growth deficits in *polq* mutants. Just recently, it was shown that developmental deficiencies are related to endogenous replicative stress, explaining the growth variability (Nisa et al., 2021). The simultaneous loss of AtPOLQ and AtKU70 almost abolished root growth (Figure 1C). On average, the root length was reduced by more than 90% compared to wildtype plants. This synergistic effect suggests that AtPOLQ and AtKU70 act within parallel pathways to repair aberrant DNA replication intermediates in the root meristem and that NHEJ is especially crucial for root proliferation.

The *ku70/polq* double mutant shows chromosomal fragmentation due to deficient DSB repair in somatic cells

During the analysis of the *ku70/polq* double mutant, we also noticed growth defects during shoot, leaf, and flower development, as well as a mild reduction of fertility. In both single mutants, *ku70* and *polq*, a slightly reduced number of seeds per silique was observed (Figure 1D). In addition, anther development was partly disturbed, resulting in less pollen. Nonetheless, all pollen were viable as determined by Alexander staining (Figure S1) (Alexander, 1969).

All of these results indicated that loss of NHEJ leads to a general proliferation deficiency during somatic growth. We also expected this loss to be responsible for the reduced fertility. Therefore, we checked whether DNA damage was visible in the pre-meiotic germline of the double mutant. Mitotic replication defects are often correlated with aberrant chromosome structures in the anaphase of mitosis. To test our hypothesis, mitotic anaphases of somatic anther cells were analyzed. Almost no defects were detected in cells of wildtype plants as well as in the mutant line *ku70* (Figure 2a). In contrast, loss of AtPOLQ resulted in an increase of chromosomal breaks which were detected in about 1.3% of the analyzed cells. The simultaneous defect in both AtPOLQ and AtKU70 resulted in a much stronger effect: approximately 5.5% of the cells showed abnormalities during the progression of mitosis (Figure 2b). In general, these abnormalities are either bridges between chromosomes, indicating a mis-repair of

(a)

	total number of analyzed anaphases	number of chromosome fragmentations	number of anaphase bridges	defects in mitotic anaphases (%)
WT	316	1	1	0.63
ku70	309	1	1	0.65
polq	315	3	1	1.27
ku70/polq	329	13	4	5.17

(b)

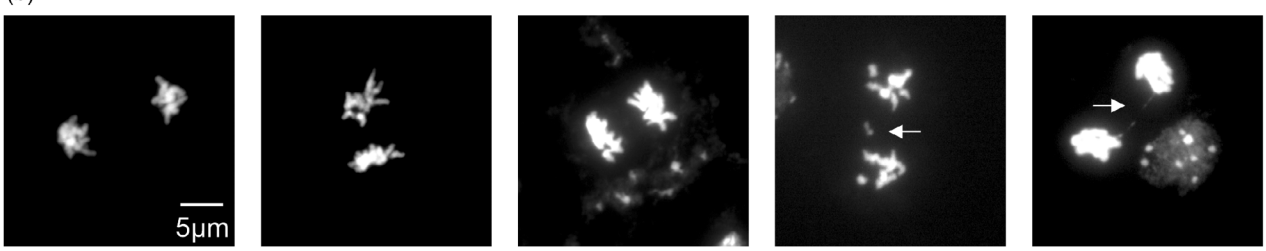


Figure 2. Mitotic anaphase defects in non-homologous end-joining (NHEJ) mutants compared to wildtype.
(a) Listed are the number of defects (chromosome fragmentations and anaphase bridges) detected within mitotic anaphases of the *ku70/polq* double mutant compared to single mutants and wildtype (WT) plants. Additionally, the total number of all examined nuclei is portrayed.
(b) Mitotic anaphases were analyzed by performing DAPI staining of chromosome spreads. The *ku70/polq* double mutant revealed more defects (anaphase bridges or chromosome fragmentations) in mitotic anaphases compared to the respective single mutants or wildtype.

DSBs, or chromosomal fragments, originating from a lack of DSB repair. Interestingly, chromosomal fragments were detected in about three-quarters of the cases, suggesting that DSB repair is severely impaired in somatic cells. These observations also suggest pre-meiotic defects of DSB repair as the ultimate cause for the reduced fertility phenotype of the *ku70/polq* double mutant.

AtPOLQ and AtKU70 act in parallel pathways of DSB repair

In addition, we were interested in testing directly how the simultaneous elimination of both factors will affect the ability of the corresponding plants to repair DSBs. Therefore, 1-week-old plants of the individual lines were treated with the genotoxin bleomycin which induces random DSBs (Haidle, 1971; Suzuki et al., 1969). After an incubation period of 2 weeks, the fresh weight of the treated plantlets was determined and related to the untreated controls of the same lines. None of the single mutants showed hypersensitivity to the DSB-inducing agent bleomycin. In contrast, a significantly reduced relative fresh weight was detected in the *ku70/polq* double mutant already at a concentration of 0.05 mg ml^{-1} bleomycin compared to wildtype plants and the corresponding single mutants (Figure 3). This indicates that both NHEJ pathways are not only capable of repairing bleomycin-induced DSBs but can also efficiently complement each other in this type of damage.

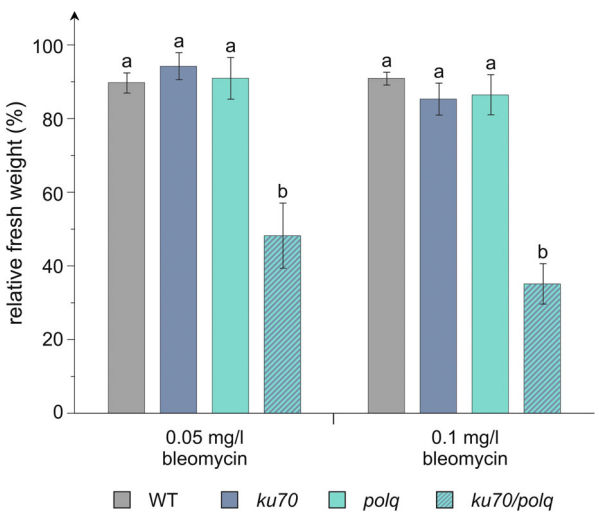


Figure 3. Sensitivity analysis of non-homologous end-joining (NHEJ) mutants compared to wildtype in response to bleomycin.
The fresh weight of the double mutant *ku70/polq* and the corresponding single mutants as well as wildtype (WT) plants was determined 13 days after genotoxin induction. The relative fresh weight was calculated using untreated control samples of each line. Shown is the average relative fresh weight of at least three independent assays. Error bars correspond to standard deviations. After treatment with 0.05 and 0.1 mg ml^{-1} bleomycin, a significantly reduced relative fresh weight of the *ku70/polq* double mutant was observed compared to the two single mutants and wildtype. The statistical significance was determined via Fisher LSD test (*P*-value <0.0001), with different lowercase letters representing significant differences.

Agrobacterium-mediated GT efficiencies can be enhanced in AtKU70- but not in AtPOLQ-deficient plants

As the two NHEJ pathways appear to complement each other, at least in bleomycin-induced DSBs, the double mutant posed a promising candidate for CRISPR/Cas-mediated GT. Therefore, we hypothesized that complete elimination of HR-competitive NHEJ would result in preferred DSB repair by HR and subsequently, higher GT efficiencies.

To test our hypothesis, we were highly interested in performing GT experiments in the above-characterized *ku70/polq* double mutant. A few years ago, we established the *in planta* GT (ipGT) system, targeting the gene of acetolactate synthase (ALS) of Arabidopsis (Fauser et al., 2012; Wolter et al., 2018; Wolter & Puchta, 2019). The system is based on the simultaneous excision of a transgenic donor template and DSB induction in the target locus in the egg cell of T1 plants (Figure 4a). T2 plants are then screened for imazapyr-resistant seedlings that represent GT events. The highest ipGT frequencies could be obtained by the use of the highly efficient temperature-tolerant LbCas12a (Merker et al., 2020; Schindele et al., 2023; Schindele & Puchta, 2020).

To be able to evaluate the differences in GT efficiencies, the experiments were performed using both the double and single mutants and the wildtype. As stable transformation of POLQ-deficient Arabidopsis plants is not possible (van Kregten et al., 2016), we could not perform the ipGT assay for this evaluation. Instead, GT efficiencies were already evaluated in the T1 generation (Figure 4b). This setup is comparable to GT experiments performed by the transformation of various types of somatic plant cells in tobacco seedlings (Huang & Puchta, 2019) or rice calli (Li et al., 2020).

For this experiment, the plants were transformed with the GT plasmid carrying the Cas12a ORF (Figure 4a) via floral dipping (Clough & Bent, 1998). As in this experimental approach, the GT efficiency is much lower than in the ipGT approach, multiple transformations had to be performed to obtain a sufficient number of seeds to be able to screen for GT events. The number of transformed plants and the number of analyzed seeds are listed in Figure 5C.

Since the evaluation of GT efficiencies is based on the number of seeds, the respective seed weights of the wildtype and mutants were determined beforehand to be able to estimate the number of seeds by weight. However, no significant differences in weight were observed (Figure S2) between the different mutant phenotype and wildtype seeds. In order to evaluate GT efficiencies accurately, it was also necessary to evaluate the germination rate of the mutant and wildtype seeds. The germination rate was

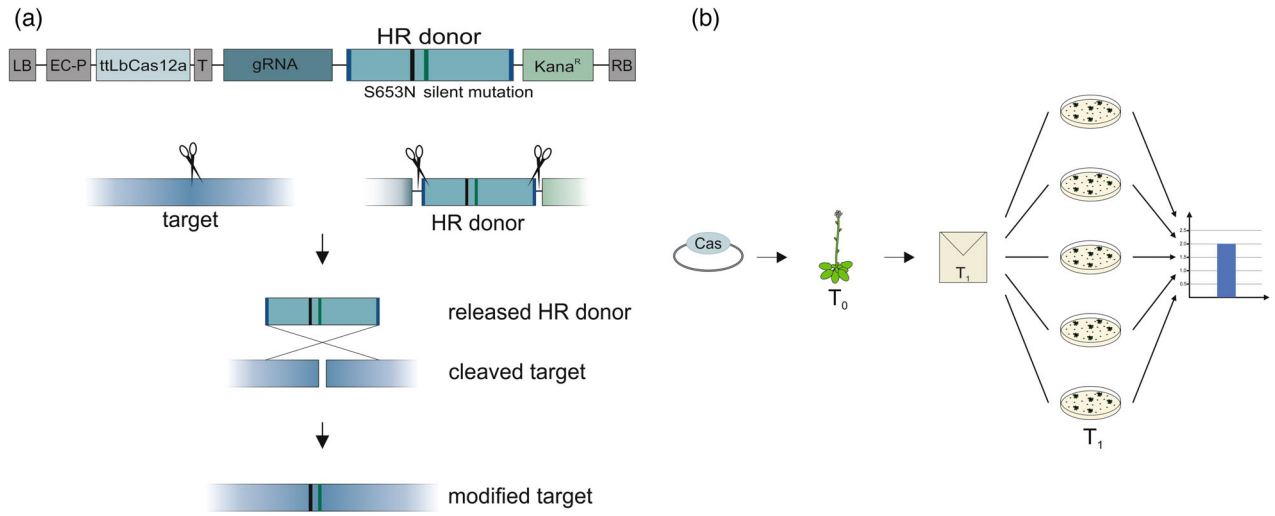


Figure 4. Procedure of gene targeting (GT) experiments.
(a) The GT construct comprises sequences flanked by the left and right border (LB and RB). It contains the ttLbCas12a expression cassette, which consists of an egg cell-specific promoter (EC-P), the ttLbCas12a sequence, and a rbcS terminator (T). Additionally, it contains a guide RNA (gRNA) expression cassette consisting of a gRNA sequence regulated by a U6-26 promoter and a PolyT termination signal. The gRNA contains the spacer sequence to guide the Cas nuclease to the target sequence. The gRNA expression cassette is followed by the sequence of the HR donor, which comprises a sequence homologous to part of the *ALS* gene, flanked by protospacer sequences. In addition, the donor contains a mutation (S653N) that enables selection for herbicide resistance as well as a silent mutation that prevents donor cleavage.
(b) Arabidopsis T0 plants are transformed via floral dipping using the GT construct. Plants are cultivated to seed maturity and subsequently harvested. T1 seeds are sown on herbicide-containing medium and cultivated for 2 weeks. GT efficiency can then be determined by calculating the ratio of viable GT plants to the number of germinating seeds.

significantly lower in the double mutant which showed a reduction by approximately 50% (Figure 5A). After the T1 seeds had been incubated for 2 weeks at 22°C on herbicide-containing medium, the average GT efficiency was conducted and normalized by the germination rate.

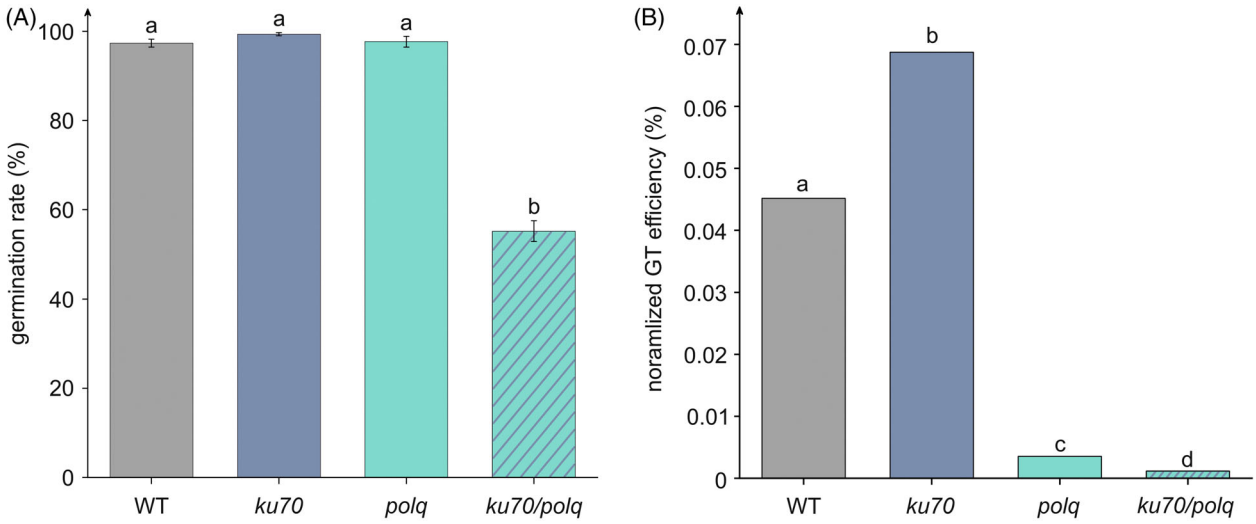
The average GT efficiency in wildtype was 0.0452% and could be increased by 1.5-fold by performing the experiment in *ku70*. Surprisingly, in AtPOLQ-deficient plants, GT efficiencies were dramatically decreased. Only few viable GT plants were detected in the approaches using AtPOLQ-deficient plants. In *polq* single mutants, GT efficiencies were reduced by factor 15 compared to wildtype. Even more surprising was that the lowest GT efficiencies were observed in the double mutant. In these plants, the GT efficiency was reduced by factor 40 compared to wildtype (Figure 5B).

To confirm the phenotype-based GT events at the molecular level, PCR-based analyses were performed using gDNA of the viable T1 plants (Figure 5D). Molecular analyses revealed a rate of perfect GT events of around 70% for all approaches, consistent with previous ipGT results. Furthermore, homozygous GT events were detected in the first generation, which had also been the case in previous ipGT approaches (Merker et al., 2020; Wolter et al., 2018; Wolter & Puchta, 2019). These homozygous events were identified by a homozygous sequencing result.

In AtPOLQ-deficient plants, GT efficiencies can only be evaluated after transient transformation in the T1 generation. In contrast, in *ku70* and wildtype plants, determination of ipGT efficiency is possible in the T2 generation due to stable transformation. In order to test whether, and how efficient, a knock out of cNHEJ would enhance ipGT frequencies, targeting was performed in the *ku70* mutant in comparison to wildtype. The results demonstrate that the ipGT approach is far more efficient than the transient GT approach – already in wildtype by an order of magnitude. Similar to the GT approach in the T1 generation, ipGT in the *ku70* mutant was more efficient than in wildtype (Figure 6a). However, the enhancement was much more pronounced: while an average efficiency of 0.5% was observed in wildtype, the average efficiency in the *ku70* mutant was more than eight times higher and reached almost 4% (Figure 6b). This is a notable increase that makes the *ku70* mutant a very attractive tool for performing GT experiments using the *in planta* approach. The obtained GT events were confirmed by conducting molecular analyses, revealing that most events resulted from perfect homologous integration (Figure 6c).

DISCUSSION

POLQ is a unique protein that is highly conserved. However, its function varies greatly between different organisms (Beagan & McVey, 2016). In this study, we could further characterize the biological functions of AtPOLQ and were able to show that it indeed plays a central role in DSB repair in a parallel pathway to cNHEJ in *Arabidopsis thaliana*.



(C)

	number of transformed T0 plants	number of analyzed T1 seeds	number of germinating T1 seeds	number of GT plants	normalized GT efficiency (%)
WT	220	~790,000	~766,000	346	0.04517
ku70	200	~389,000	~386,000	269	0.06965
polq	700	~1,630,000	~1,596,000	52	0.00307
ku70/polq	964	~510,000	~256,000	3	0.00112

(D)

	number of analyzed T1 plants	number of analyzed T1 plants with GT event	perfect GT events (%)	number of homozygous GT events	homozygous GT events (%)
WT	49	38	77.55	5	10.20
ku70	48	34	70.83	1	2.08
polq	7	6	85.71	-	-
ku70/polq	3	2	66.67	1	33.33

Figure 5. Gene targeting (GT) results of non-homologous end-joining (NHEJ) mutants compared to wildtype in the T1 generation.
(A) Shown is the germination rate of seeds of the NHEJ mutants and wildtype (WT). The germination rate of wildtype is 97%, that of *ku70* 99%, that of *polq* 98% and that of the double mutants 55%. The statistical significance was determined via the Fisher LSD test (*P*-value <0.0001), with different lowercase letters representing significant differences.
(B) The graph shows the GT efficiency evaluated in the T1 generation of the NHEJ mutants and wildtype. The efficiency of wildtype is 0.04517%, that of *ku70* 0.06965%, that of *polq* 0.00307%, and that of the double mutant 0.00112%. The statistical significance was determined via the chi-square test (*P*-value <0.0001), with different lowercase letters representing significant differences.
(C) Listed are the quantitative numbers of the GT experiment. Shown is the number of analyzed T0 plants, the number of analyzed T1 seeds, the number of germinating T1 seeds, the number of viable GT plants, and the normalized GT efficiency.
(D) Listed are the quantitative numbers of the molecular analyses. Shown is the number of analyzed T1 plants, the number of plants that showed GT events, the rate of perfect GT events, the number of homozygous GT events, and the rate of homozygous GT events.

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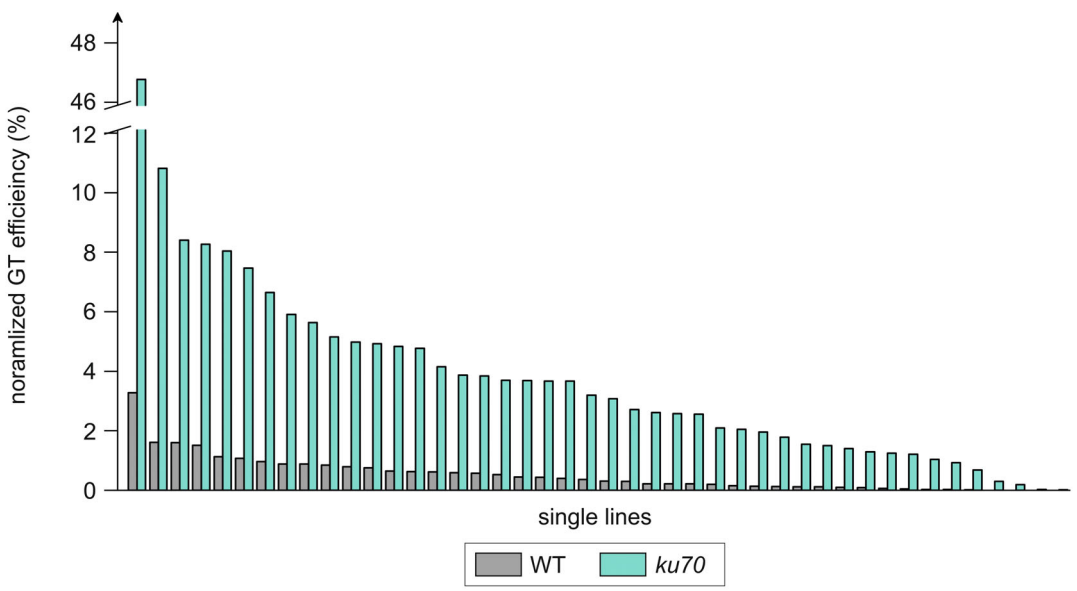
(a)



(b)

	number of analyzed T1 lines	number of positive T1 lines	positive lines (%)	GT efficiency (%)	normalized GT efficiency (%)	number of T1 GT events
WT	51	40	78.43	0.45	0.47	-
ku70	50	44	88.00	3.90	3.93	1

(c)

	number of analyzed T2 plants	number of analyzed T2 plants with GT event	perfect GT event (%)	number of homozygous GT events	homozygous GT events (%)
WT	24	18	75.00	-	-
ku70	52	47	90.38	5	9.62

Figure 6. *In planta* gene targeting (ipGT) results of the *ku70* single mutant compared to wildtype.
(a) Shown is the ipGT efficiency of every single line of the *ku70* mutant compared to those of the wildtype. The highest GT efficiency was determined in a line of *ku70*, reaching a value of 47.7%. The average GT efficiency is 0.47% in the case of wildtype and 3.93% in the *ku70* single mutant.
(b) Listed are the quantitative numbers of the T2 ipGT experiment with the number of analyzed T1 lines, the number of positive T1 lines, the proportion of positive lines, the GT efficiency, the normalized GT efficiency, and the number of T1 GT events.
(c) Listed are the quantitative number of the T2 molecular analyses, including the number of analyzed T2 plants, the number of plants with GT events, the proportion of perfect GT events, the number of homozygous GT events, and their proportion.

cNHEJ and aNHEJ are able to complement each other efficiently in DSB repair in plants

By simultaneous mutation of At*POLQ* and At*KU70*, the two most important pathways of DSB repair in somatic cells can be blocked. We were interested to investigate whether such a double mutant would be viable in plants at all, as no characterization of a viable organism has yet been published in regard to mammals. Using mouse cells, it has

been shown that simultaneous loss of these two factors leads to impaired cell proliferation and increased chromosome aberrations (Wyatt et al., 2016). In our experiments, we have now been able to demonstrate that plants lacking both genes are viable and fertile. However, compared to wildtype and the respective single mutants, the corresponding individuals show severe growth defects, they are much smaller and have deformed leaves. Moreover, the root length is drastically reduced, indicating that cell replication in the root meristem has been massively hampered (Curtis & Hays, 2007). Similar results were just recently reported in an independent study, although no further characterization of the double mutant was performed (Lv et al., 2022).

During genomic DNA replication, DSBs can arise, either if aberrant replication intermediates are processed nucleolytically or during replication from unrepaired single-strand breaks (SSBs) (Kruisselbrink et al., 2008; Paeschke et al., 2011). The occurrence of such DSBs can result in cell cycle delay and growth arrest. Recently, POLQ was shown to be involved in the repair of double-ended DSBs, resulting from SSBs originating from collapsed replication forks in human cell cultures during replication. This was confirmed by the detection of an increased sensitivity of POLQ-deficient cancer cells to topoisomerase-inhibiting agents which induce SSBs at replication forks. Moreover, loss of POLQ resulted in hypersensitivity to replication stress-inducing agents, such as hydroxyurea or ATR inhibitors (Wang et al., 2019). Additionally, recent results in Arabidopsis indicate an important role of AtPOLQ in replicative repair (Hacker et al., 2022; Nisa et al., 2021).

Our data show that NHEJ-mediated DSB repair appears to be particularly crucial for root proliferation. Moreover, our cytological analysis demonstrates that both cNHEJ and aNHEJ complement each other quite efficiently in suppressing the arising of unrepaired DSBs (Figure 2). Whereas very few broken chromosomes were detected in the single mutants, the number was increased by almost an order of magnitude in the double mutant. That the two pathways can complement each other in DSB repair was confirmed by the fact that the double mutant, but neither single mutant, showed sensitivity to the DSB-inducing agent bleomycin. Nevertheless, plant cells can also survive in absence of both NHEJ pathways. This indicates that arising DSBs must be repaired by other pathways. On one hand, this can be achieved by the use of homologous sequences. The classical HR machinery might either use the replicated sister chromatid (Vu et al., 2014) or an ectopic sequence (Puchta, 1999) as a repair template via the synthesis-dependent strand annealing mechanism (Puchta, 1998). If breaks occur within tandem repeats, they can be efficiently repaired by single-strand annealing (Siebert & Puchta, 2002). Moreover, there are indications that a further backup pathway of DSB repair might exist in plant cells (Charbonnel et al., 2011). Furthermore, it seems that plants are generally better equipped to survive major deficiencies in DSB repair than mammals. This also holds true for the factor BRCA2 which is central for the progression of HR-mediated DSB repair. Whereas the knockout phenotype is embryo-lethal in mice (Sharan et al., 1997), viable but sterile individuals were obtained in the case of Arabidopsis (Seeliger et al., 2012). A reason for this might be that the presence of an efficient elimination mechanism of severely genome-damaged cells is necessary for cancer suppression in mammals.

Loss of AtPOLQ has a dominant-suppressive effect on *Agrobacterium*-mediated GT efficiency

To overcome low HR frequencies hindering efficient GT, the imbalance of NHEJ and HR poses a major obstacle. Shifting this imbalance toward HR by blocking cNHEJ has already proven to be a promising strategy in plants and mammals (Fattah et al., 2008; Qi et al., 2013). Qi et al. (2013) detected not only higher HR rates by knocking down cNHEJ in Arabidopsis but also increased numbers of larger deletions at the DSB induction site which can be attributed to aNHEJ. Similar results were obtained in rice (Nishizawa-Yokoi et al., 2012). This suggests that, by knocking down one DSB repair pathway, the rates of the others increase. Since we were able to show by two different experimental approaches that aNHEJ and cNHEJ can complement each other efficiently in DSB repair (Figures 2 and 3), the use of a double mutant seemed to be a promising genetic background for GT approaches. Therefore, we performed GT experiments in Arabidopsis by *Agrobacterium*-mediated delivery of the DNA template, together with egg cell-specific expression of ttLbCas12a by floral dip transformation. Although the overall targeting frequencies were an order of magnitude lower than in the ipGT approach, we were able to show that GT efficiency is enhanced in cNHEJ mutant backgrounds. As already shown by others (Nishizawa-Yokoi et al., 2012; Qi et al., 2013), knockout of cNHEJ helps to enhance GT frequencies. In our case, the effect was especially significant when applied to the ipGT strategy.

In contrast, we found no indication that knockout of *POLQ* enhances *Agrobacterium*-mediated GT frequencies. This finding is in line with a study of van Tol et al. (2022) which showed that Arabidopsis *polq* mutants can be used for GT experiments but with very low efficiency. In their study, they obtained two heritable GT events in the *polq* mutant background, which was about an order of magnitude less events than in wildtype. However, due to the small number of GT events, it was difficult to conclude whether GT efficiency is indeed reduced in the mutant. In our experiments, we obtained 52 independent GT events in the *polq* mutant and the GT frequency was one and a half orders of magnitude lower than in the wildtype

background. Therefore, we now can conclude with a high degree of certainty that the knockout of *POLQ* leads to a severe reduction in *Agrobacterium*-mediated GT efficiency in plants.

Moreover, in the double mutant, the GT-enhancing effect of KU70 loss is completely suppressed by the additional loss of POLQ. This, again, is astonishing as the number of GT events was not reduced in the absence of POLQ in mammalian cells in comparison to wildtype in several studies (Arai & Nakao, 2021; Saito et al., 2017; Zelensky et al., 2017). In fact, even the opposite effect was reported recently using human cells: simultaneous elimination of cNHEJ and aNHEJ led to an increased GT efficiency (Wimberger et al., 2023).

For this very surprising phenomenon, there are two possible hypotheses.

On the one hand, in contrast to mammals (Mateos-Gomez et al., 2015), one could speculate that POLQ might play an active role in a HR-mediated repair of DSBs in plants. Indeed, there are reports that point in this direction. Using an intrachromosomal homologous recombination assay, Inagaki et al., 2006 were able to demonstrate that, in Arabidopsis, knockdown of *POLQ* is correlated with a deficiency of HR. Also, in the model algae *Chlamydomonas*, two independent studies showed that POLQ is required for CRISPR/Cas-mediated HR-based GT (Ferenczi et al., 2021; Sizova et al., 2021).

On the other hand, the low *Agrobacterium*-mediated GT efficiency in POLQ-deficient plants might be caused by an indirect effect. As the PolQ mutant is T-DNA integration-deficient, the number of available template molecules for HR as well as for the expression of the Cas nuclease might be drastically reduced in comparison to wildtype. In order to test this hypothesis, plants with stably integrated T-DNA would first have to be established and *POLQ* subsequently knocked out to generate a POLQ-deficient plant with integrated T-DNA. This is exactly what was accomplished recently by the group of Marcel Tijsterman and Paul Hooykaas (see accompanying report: Kralemann et al., 2023). Impressively, this approach resulted in an increased GT efficiency, indicating that the effect of POLQ on HR is indeed indirect. The fact that the GT efficiency was even more reduced in the double mutant compared to the *polq* single mutant in our experiments, might also be due to the accumulation of further DSBs: The lack of NHEJ means that these breaks might be repaired mainly by HR using the sister chromatid, which could significantly reduce the enzyme activity available for homologous recombination at the target site in comparison to the wildtype situation.

Although we did not achieve our goal of enhancing GT efficiency in Arabidopsis by knocking out both NHEJ pathways, our improvement of the technology by using the ipGT strategy (Fauser et al., 2012) in combination with the knockout of cNHEJ is notable. We achieved an about eightfold enhancement of ipGT in comparison to wildtype. In almost a quarter of the progeny of individual T1 lines, the rates of positive seedlings were above 5%. This high efficiency was also documented by the fact that several lines homozygous for the GT event were found in the *ku70* mutant. Thus, using ipGT in the *ku70* mutant background might be a highly promising approach for practical applications, not only in Arabidopsis.

MATERIALS AND METHODS

Plant material and growth conditions

All *A. thaliana* lines used in this study were of the Columbia (Col-0) background. The mutant lines *polq* (*teb-5*, SALK_018851) and *ku70* (*ku70*-1, SALK123114) have been described previously (Inagaki et al., 2006; Jia et al., 2012). The analyzed double mutants were generated by crossbreeding of homozygous T-DNA insertion lines. Their homozygosity was determined in the F2 generation by PCR-based genotyping, using wildtype- and T-DNA-specific primer combinations (Tables S1 and S2). Plants were either cultivated in a greenhouse on soil (1:1 mixture of Floraton 3 [Floragard, Oldenburg, Germany] and vermiculite [2–3 mm; Deutsche Vermiculite Dämmstoff, Sprockhövel, Germany]) at 22°C (16 h light/8 h dark) or on agar plates in growth chambers (CLU-36L4; Percival Scientific, Perry, IA, USA) under stable and axenic conditions (16 h light at 22°C/8 h dark at 22°C). For growth under axenic conditions, the seeds were surface-sterilized (4% sodium hypochlorite), rinsed in water, and stratified (overnight at 4°C), before sowing on agar plates containing germination medium (GM, 4.9 g L^{-1} Murashige and Skoog, 10 g L^{-1} sucrose and 7.6 g L^{-1} agar; pH 5.7).

Root growth assays

The sterilized and stratified seeds were sown in a row on GM (1% agar) in square plates. The plates were incubated vertically in a growth chamber. After 9 days of incubation, images of the roots were taken and evaluated using ImageJ and the Add-on SmartRoot (Lobet et al., 2011). At least three biological replicates were analyzed and the root length of each of at least seven plants per replicate was determined.

Fertility assays

To study the fertility of the mutant lines, the plants were grown in the greenhouse for about 4 weeks. Five mature siliques from at least five different plants were decolorized overnight in 70% ethanol. The number of seeds per silique was determined using a binocular microscope.

Alexander staining of 4-week-old plants was performed to investigate pollen viability. Closed buds were fixed for 1 h in Carnoy solution (ethanol [99.5%]:chloroform:acetic acid [100%]; proportionally 6:3:1). Subsequently, the buds were washed with deionized water and opened. The anthers were dissected and transferred to microscope slides. By adding a staining solution (0.5 ml ethanol [95%], 50 µl Malachite green [1% in ethanol], 1.25 ml glycerol, 0.25 g chloral hydrate, 250 µl fuchsic acid [1% w/v in deionized water], 25 µl Orange G [1% w/v in deionized water], and 200 µl acetic acid in 5 ml deionized water), live pollen were stained red/purple and dead ones blue. After incubation overnight at 60°C, the evaluation was carried out using a binocular microscope and digital imaging. At least 20 different anthers of each line were analyzed.

DAPI staining of mitotic chromosomes

Chromatin preparation of mitotic cells was performed analogously to the preparation of male meiocytes as described previously (Armstrong et al., 2009).

Sensitivity assays

To analyze the sensitivity of the different Arabidopsis mutant lines to the genotoxin bleomycin, assays were performed as previously described (Dorn & Puchta, 2020). Arabidopsis seeds were sterilized with 4% sodium hypochlorite, stratified overnight, and sown on agar plates containing GM media. After 7 days, plants were transferred to six-well plates containing 4 ml (control batch without genotoxin) or 5 ml of liquid GM medium. On the following day, 1 ml of the different genotoxin concentrations was added. The used genotoxic agents were dissolved in sterile ultrapure water. After a further 13-day incubation period, the relative fresh weight was determined. The values of the treated samples were normalized using the untreated control samples of the corresponding line. At least three independent biological replicates were analyzed per assay.

GT experiments

For the GT experiments, the ttLbCas12a construct described in Merker et al. (2020) was used. For transient transformation, *Agrobacterium tumefaciens*-mediated Arabidopsis transformation was performed according to the 'Floral Dip' method of Clough and Bent (1998). This step was repeated three times with the wildtype, six times with the *ku70* single mutant, seven times with the *polq* single mutant, and 10 times with the double mutant. After harvesting the T1 seeds, they were weighed to determine the total number of seeds. Then, the number of germinating seeds was determined by relating the total number of seeds to the germination rate. Afterward, the seeds were surface-sterilized and stratified overnight. Seeds were then sown on imazapyr-containing (5 μM) GM medium and cultivated for 2 weeks in a growth room under stable and axenic conditions (16 h light at 22°C/8 h dark at 22°C). Subsequently, GT efficiency was determined by relating the number of viable GT plants to the ratio of germinating seeds. To confirm GT events on the molecular level, genomic DNA of GT plants was extracted following the protocol of Edwards et al. (1991). Subsequently, a PCR was performed using the primers FW183 and FW58 (Table S2) to amplify the modified sequence. To prevent amplification of the donor sequence, primer binding sites were placed outside of the donor sequence. The amplicon was then purified and analyzed by Sanger sequencing to determine the ratio of perfect GT events.

ipGT experiments

For the ipGT experiments, a construct similar to that described in Merker et al. (2020) was used. The construct contained a slightly different donor template with an additional silent mutation and a gentamicin resistance cassette instead of a kanamycin resistance cassette. The construct was stably transformed via the *A. tumefaciens*-mediated Arabidopsis transformation (Clough & Bent, 1998). T1 seeds were harvested, primary transformants were selected and transferred to the greenhouse until seed maturity. Then, the number of germinating T2 seeds was determined by relating the total number of seeds to the germination rate. Afterward, seeds were sterilized, stratified, sown on imazapyr-containing GM medium, and cultivated for 2 weeks in a growth room (16 h light at 22°C/8 h dark at 22°C). Then, the GT efficiency of each T1 line was calculated by relating the number of viable GT plants to the ratio of germinating seeds. Additionally, molecular analyses were performed in the same manner as described above.

ACCESSION NUMBERS

Sequence data of the T-DNA insertion lines from this article can be found at The Arabidopsis Information Resource (TAIR) using the following accession numbers: *A. thaliana POLQ*: At4g32700, *A. thaliana KU70*: At1g16970 (Alonso et al., 2003; Kleinboelting et al., 2012).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

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

Figure S1. Pollen viability of NHEJ mutants compared to wildtype. Pollen viability was analyzed by performing Alexander staining. In none of the tested lines dead pollen were detected. Shown are representative pictures of one anther per line. Approximately 30% of the *ku70/polq* double mutant anthers were strongly deformed and contained less pollen than the corresponding single mutants or wildtype (WT) plants (scale bar = 100 μm, $n = 28$).

Figure S2. Seed weight of NHEJ mutants compared to wildtype. Shown is the determined seed weight of wildtype (WT) and of the NHEJ mutants. The average seed weight of the wildtype is 21.52 μg, that of the *ku70* single mutant is 21.24 μg, that of the *polq* single mutant is 23.53 μg and that of the *ku70/polq* double mutant is 23.16 μg. The statistical significance was determined via Fisher LSD test, P-value <0.01.

Table S1. Primer combinations for genotyping.

Table S2. Sequences of the oligonucleotides used in this study.

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