Improving targeted gene editing in Arabidopsis thaliana
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Citation
Kamoen, L. (2025, June 10). *Improving targeted gene editing in Arabidopsis thaliana*. Retrieved from https://hdl.handle.net/1887/4249642

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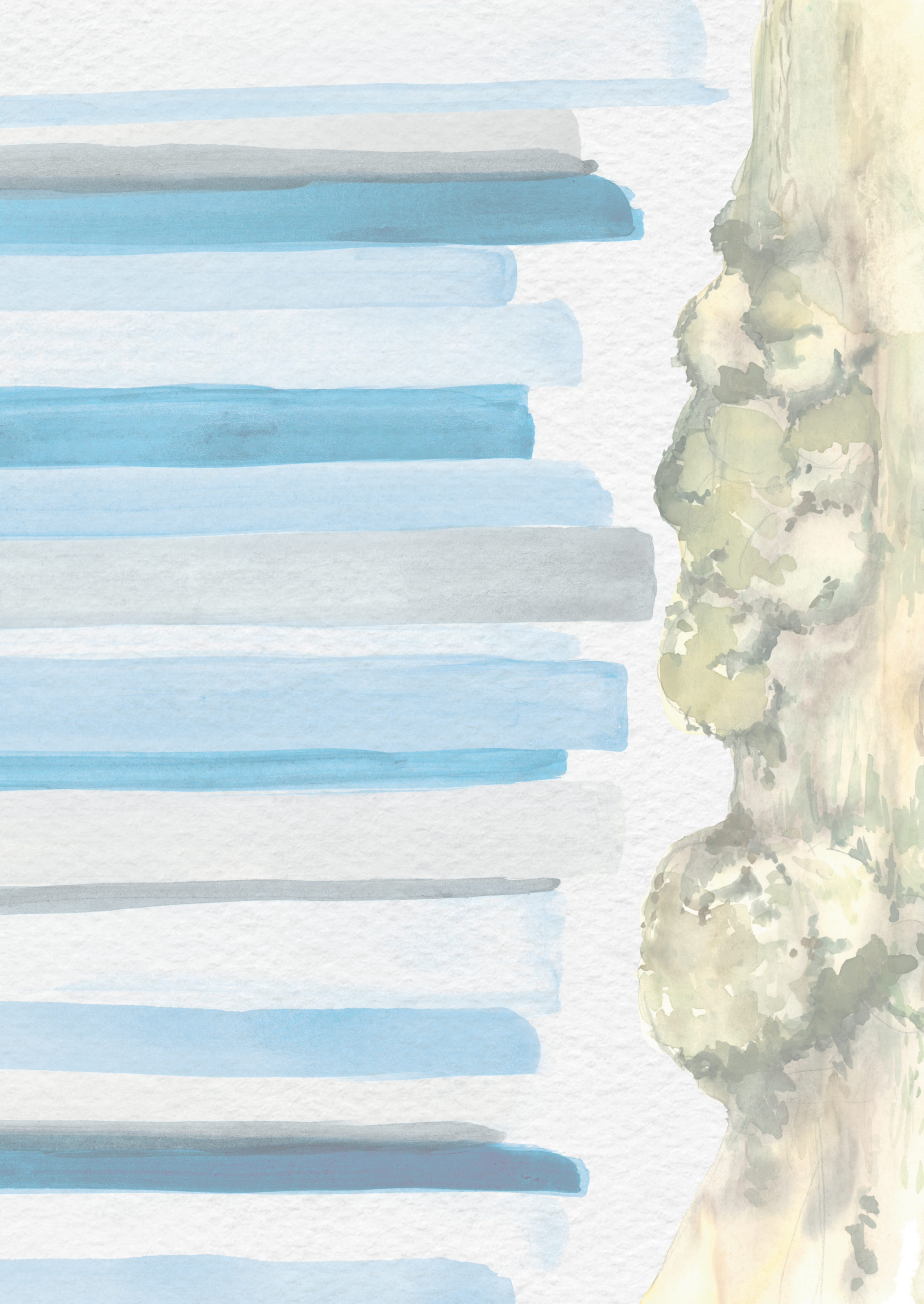

Chapter 1

General introduction

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Double strand break repair

DNA integrity is crucial for cellular function and survival, yet it is constantly threatened by various forms of damage, with double-strand breaks (DSBs) being particularly detrimental. These breaks can arise from various sources, including ionizing radiation, chemical exposure, and internal cellular processes (Jackson, 2002). DSBs are especially severe because they disrupt both strands of the DNA helix, complicating the repair process. If not accurately repaired, DSBs can result in chromosomal aberrations, genomic instability, mutations, accelerated aging, and cell death (Van Gent *et al.*, 2001). To mitigate these threats, cells have evolved sophisticated DNA repair mechanisms. Upon detecting DNA damage, cellular responses activate cell-cycle checkpoints, halting the cell cycle to provide time for repair. The DNA damage response (DDR) not only facilitates DNA repair but also triggers apoptosis or senescence if the damage is irreparable (Lawrence *et al.*, 2015).

DSB repair in higher eukaryotes primarily occurs through two pathways: homologous recombination (HR) and end joining (EJ) (Figure 1). HR is an error-free repair mechanism using a homologous sequence, often the sister chromatid, as a template. The process involves end resection to generate single-stranded DNA (ssDNA) overhangs, which are coated by Replication Protein A (RPA) which is later replaced by RAD51 (Eschbach & Kobbe, 2014; Mimitou & Symington, 2009; Wang & Haber, 2004). RAD51 facilitates homology search and DNA strand invasion, leading to DNA synthesis and repair (Symington *et al.*, 2014). HR can proceed through several sub-pathways, including the Holliday Junction (HJ) pathway and synthesis-dependent strand annealing (SDSA) (Elbakry & Lobrich, 2021).

In contrast to HR, EJ directly joins DNA ends without a template. EJ includes classical nonhomologous end joining (cNHEJ), polymerase theta-mediated end joining (TMEJ), extended microhomology-mediated end joining (eMMEJ), and single-strand annealing (SSA).

In cNHEJ, the Ku70/80 complex binds to DSB ends, preventing degradation and recruiting DNA ligase IV (Lig4) for ligation (Chang *et al.*, 2017; Deriano & Roth, 2013). While cNHEJ is often precise, it can result in small insertions or deletions (Betermier *et al.*, 2014).

TMEJ, often referred to as microhomology-mediated end joining (MMEJ), uses microhomology to anneal and prime DNA synthesis (Ozdemir *et al.*, 2018; Wood & Doublie, 2016; Wyatt *et al.*, 2016). TMEJ relies on DNA polymerase theta (Polθ), which consists of three main domains: an N-terminal helicase-like domain, a central unstructured domain, and a C-terminal polymerase domain (Beagan *et al.*, 2017; Seki *et al.*, 2003). The N-terminal helicase-like (Polθ-hel) domain exhibits ATPase activity and is involved in unwinding DNA (Mateos-Gomez *et al.*, 2017; Newman *et al.*, 2015; Ozdemir *et al.*, 2018; Vanson *et al.*, 2022). The central unstructured domain provides flexibility and may facilitate interactions with other proteins (Yousefzadeh & Wood, 2013). The C-terminal polymerase domain (Polθ-pol) is responsible for the DNA synthesis activity of Polθ (He & Yang, 2018; Kent *et al.*, 2015). In TMEJ, Polθ-hel facilitates annealing of the microhomologous sequences by displacing other proteins such as RPA (Mateos-Gomez *et al.*, 2017; Schaub

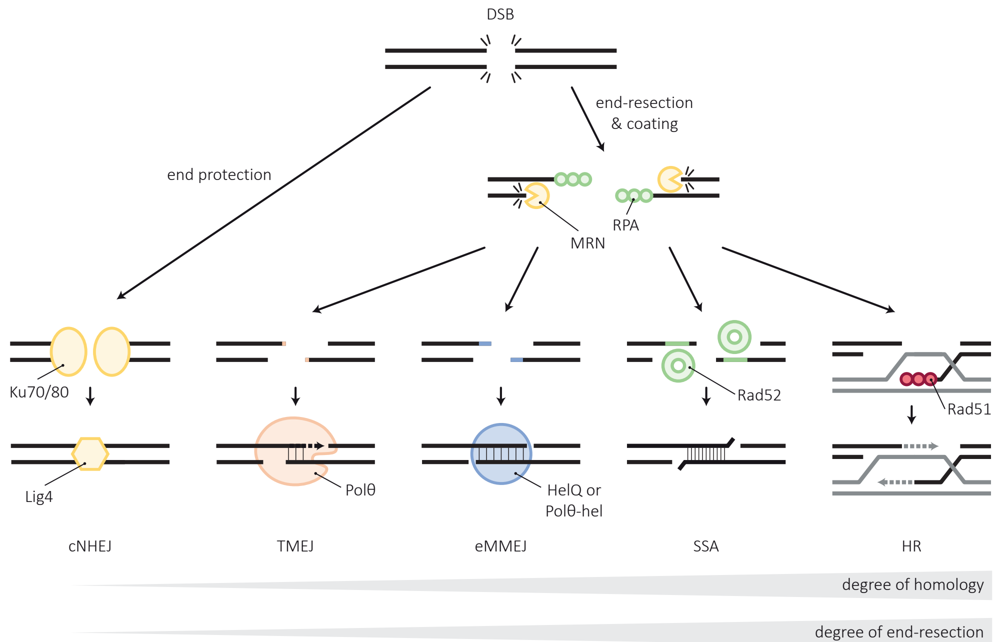


Figure 1. Overview of DNA double-strand break (DSB) repair pathways.
Classical nonhomologous end joining (cNHEJ): The Ku70/80 heterodimer recognizes the break ends and protects them from degradation. Ligase 4 (Lig4) seals the break by ligating the ends together. cNHEJ can lead to deletions or insertions depending on the condition of the break ends.
Polymerase theta-mediated end joining (TMEJ): When the DSB ends are resected, but HR is unavailable, TMEJ typically repairs the breaks. This pathway uses polymerase theta (Polθ) to anneal microhomologous sequences in the ssDNA overhangs, and extend them. This results in deletions and sometimes insertions.
Extended Microhomology-Mediated End Joining (eMMEJ): When larger microhomologous sequences are present in the flanks of the DSBs, end joining guided by these extended microhomologies is called eMMEJ. In nematodes, HelQ performs annealing of these extended microhomologies. In plants, where HelQ is absent, the helicase domain of polymerase theta (Polθ-hel) performs this task.
Single-Strand Annealing (SSA): SSA repairs DSBs using sizable repeats surrounding the break. This pathway involves resection of the break ends to expose homologous sequences. Rad52 plays a crucial role in preparing these ends for annealing, which ultimately leads to deletion of the intervening sequence.
Homologous Recombination (HR): HR involves resection of break ends by the MRN complex, which are subsequently coated with replication protein A (RPA) and then RAD51 to facilitate strand invasion and error-free repair using the sister chromatid as a template.
In this schematic illustration, the pathways are ordered based on the degree of homology and end resection. End resection is absent in classical nonhomologous end-joining, while it is extensive in homologous recombination pathways, often spanning several kilobases.

et al., 2022), allowing Polθ-pol to prime DNA synthesis, creating a stable interaction between the DNA ends (He & Yang, 2018; Wyatt *et al.*, 2016). This pathway is particularly mutagenic, producing deletions with characteristic microhomology scars and occasional templated insertions due to primer template switching (Schimmel *et al.*, 2019).

Polθ-independent MMEJ has also been described and typically requires longer microhomologies (Anand *et al.*, 2022; Kamp *et al.*, 2021; Kelso *et al.*, 2019; Ramsden *et al.*, 2022) that are referred to as extended microhomology (eMH). eMMEJ was identified as a separate pathway in the nematode *Caenorhabditis elegans*, involving HelQ (Kamp *et al.*, 2021), a helicase that has high similarity to the helicase domain of polymerase theta (Marini & Wood, 2002) and which is absent in plants.

SSA, much like TMEJ and eMMEJ, repairs DSBs by utilizing homologous repeat sequences flanking the break. These homologous sequences are generally longer, often spanning several hundred base pairs, and can be positioned further apart (Bhargava *et al.*, 2016). Rad52 facilitates the annealing of complementary single-stranded regions flanking the DSB, leading to deletions between the repeats (Kelso *et al.*, 2019).

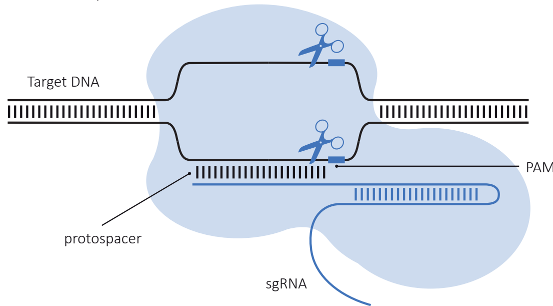
Although most research on these repair processes has been conducted in *Saccharomyces cerevisiae* (yeast) and animals such as *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (nematode worm), *Mus musculus* (mouse), and *Homo sapiens* (human) cell lines, the processes in plants are largely similar. Conversely, most of these crucial enzymes have also been identified in plants, highlighting the conservation of these processes throughout evolution. For instance, RPA (Ishibashi *et al.*, 2001), Rad51 (Doutriaux *et al.*, 1998), Ku70/80 (Tamura *et al.*, 2001), Lig4 (van Attikum *et al.*, 2003), Polθ (Inagaki *et al.*, 2006), and Rad52 (Samach *et al.*, 2011) are all present in plants. This conservation underscores the fundamental nature of these DNA repair mechanisms across different kingdoms of life.

CRISPR/Cas

Due to the error-prone nature of many of these DSB repair pathways, they are frequently harnessed to achieve mutagenesis. Historically, mutagenesis was achieved through the application of radiation or chemical agents. However, recent advancements have led to the development of site-directed break induction techniques, with the aid of zinc finger nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), which enable the precise induction of DSBs and subsequent mutagenesis at specific genomic loci.

The efficiency of site-specific mutagenesis has been significantly enhanced by the development of the CRISPR/Cas system, derived from the adaptive immune system of bacteria and archaea (Barrangou *et al.*, 2007). This system, which stands for Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins, enables precise and efficient genome editing.

The Cas protein uses RNA to locate its target site within the genome, where it induced a DSB. In the CRISPR/Cas9 system, two RNAs, crRNA (CRISPR RNA) and tracrRNA (trans-activating CRISPR RNA), form a complex with Cas9. The crRNA contains the sequence that matches the target DNA,



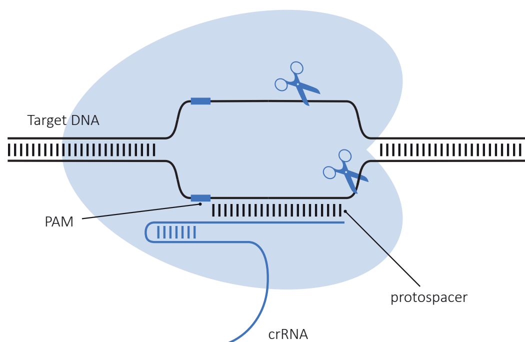


Figure 2. Schematic overview of CRISPR/Cas9 and Cas12a systems. A) Cas9 requires a single guide RNA (sgRNA) to target the DNA sequence. The sgRNA guides Cas9 to the target DNA, where it makes a double-stranded cut, resulting in blunt ends. B) Cas12a requires a crRNA for targeting. Unlike Cas9, Cas12a creates staggered cuts, producing sticky ends. Cas9 and Cas12a differ in their DNA cut types and PAM (Protospacer Adjacent Motif) requirements. Cas9 recognizes the PAM sequence NGG, while Cas12a targets T-rich PAM sequences, expanding the range of genomic sites that can be edited.

guiding the Cas enzyme to the correct location. The tracrRNA binds to the crRNA and helps the Cas enzyme recognize and bind to the target DNA. For ease of use, researchers have linked the two RNAs together into a single guide RNA (sgRNA). The sequence matching the target site is called the protospacer. This specific DNA sequence is carefully chosen to match the location in the genome where changes are desired. Adjacent to the protospacer is a short DNA sequence called the Protospacer Adjacent Motif (PAM). The PAM is crucial because it allows the CRISPR/Cas system to recognize and bind to the target DNA, enabling the Cas enzyme to make precise cuts.

Among CRISPR-associated nucleases, Cas9 and Cas12a are particularly notable for their efficiency, specificity, and applicability across various species (Swartjes *et al.*, 2020). Cas9 and Cas12a create different configurations of DSBs. Cas9 typically cuts the two DNA strands at nearly the same distance from the PAM sequence, resulting in mostly blunt DSB ends with occasionally a 1 bp stagger (Shou *et al.*, 2018). In contrast, Cas12a makes staggered cuts, with single-strand DNA incisions 4–5 bp apart, producing DNA ends with 5′ single-stranded overhangs. Unlike Cas9, Cas12a only requires a crRNA.

CRISPR/Cas technology, which allows for precise DNA cleavage at almost any location in the genome, has revolutionized genetic engineering (Jinek *et al.*, 2012). This groundbreaking work earned Emmanuelle Charpentier and Jennifer Doudna the Nobel Prize in Chemistry in 2020 for their development of this precise genome-editing method (The Royal Swedish Academy of

Sciences, 2020). The versatility and high specificity of CRISPR/Cas have made it an invaluable tool across various organisms, including plants.

CRISPR/Cas in model and crop plants

The initial demonstrations of CRISPR/Cas technology in plants were reported in a series of landmark studies (Feng *et al.*, 2013; Li *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013; Xie & Yang, 2013).

One of the plants in which CRISPR/Cas has been extensively researched and applied is *Arabidopsis thaliana*, a model organism in plant biology. *A. thaliana*, commonly known as thale cress, is a small flowering plant belonging to the Brassicaceae family, which also includes broccoli, radish, and mustard. It is a widely used model organism in plant biology and genetics due to its relatively simple genome, short life cycle, and ease of cultivation (Koornneef & Meinke, 2010). Notably, *Arabidopsis* was the first plant to have its entire genome sequenced, making it an even more valuable tool for studying plant genetics (*Arabidopsis* Genome Initiative, 2000).

Beyond research in model plants, CRISPR/Cas is increasingly being utilized to develop crops with enhanced traits, such as higher yield, improved nutritional content, and increased resistance to diseases and environmental stresses.

Notable examples of successes achieved through CRISPR/Cas technology are tomatoes resistant to parasitic weed (Bari *et al.*, 2021), bacterial speck disease (Ortigosa *et al.*, 2019), and powdery mildew (Santillan Martinez *et al.*, 2020). Additionally, various disease-resistant crops have been developed in other species, such as rice and potato resistant to bacterial blight (Blanvillain-Baufume *et al.*, 2017; Haverkort *et al.*, 2016; Oliva *et al.*, 2019).

To support the growing population amid climate change, many studies have focused on achieving crops resistant to abiotic stresses like drought and heat. For instance, drought-resistant varieties have been developed in chickpea (Badhan *et al.*, 2021), soy (Du *et al.*, 2018), rice (Lou *et al.*, 2017), and maize (Shi *et al.*, 2017). Heat-resistant varieties include rice (Qiu *et al.*, 2018) and tomato (Klap *et al.*, 2017) (see (Kumar *et al.*, 2023), and references therein).

When it comes to nutritional enhancement, examples include increased oleic acid content in peanut, enhancing both its shelf-life and nutritional value (Yuan *et al.*, 2019). Efforts are also underway to develop gluten-free wheat (Jouanin *et al.*, 2019; Sanchez-Leon *et al.*, 2018).

The work of (Zsögön *et al.*, 2018) is particularly fascinating because it demonstrates the potential of CRISPR/Cas technology to reverse the domestication process and reintroduce beneficial traits from wild species into modern crops. The domestication of tomato (*Solanum lycopersicum*) from its ancestor, *Solanum pimpinellifolium*, resulted in increased yields but also reduced genetic diversity, nutritional value, and taste. This study used CRISPR/Cas9 to create loss-of-function

alleles for four domestication traits in tomatoes. The edited plants showed up to a 200% increase in fruit weight compared to *S. pimpinellifolium*. Nutritional value also improved, with lycopene levels over 100% higher than the parent species and 500% higher than commercial cherry tomatoes. This approach demonstrates that targeted genetic engineering can rapidly create new crops. It not only paves the way for molecular breeding programs to exploit the genetic diversity present in wild plants but also highlights the potential for creating crops that are more resilient and nutritionally superior.

In Europe, CRISPR and other gene-editing technologies are currently regulated under the same stringent rules as traditional genetically modified organisms (GMOs), which imposes strict regulations on their cultivation and use (European Commission, 2018). However, there has been ongoing debate and some movement towards potentially relaxing these regulations (Callaway, 2018). Scientists and agricultural businesses have been advocating for changes, arguing that CRISPR is more precise and should be treated differently from traditional GMOs (Jones, 2015). Plants that are indistinguishable from those obtained through conventional breeding would be exempt from the stringent GMO regulations, while plants with more complex modifications would still be subject to stricter GMO rules (European Commission, 2021).

Figure 3. Oak tree infected with crown gall disease.

Agrobacterium tumefaciens

Genetically modified plants can be created using various techniques, of which *Agrobacterium tumefaciens*-mediated transformation (AMT) is the most commonly used method. AMT makes use of the soil bacterium *A. tumefaciens*, which is known for causing crown gall disease in plants (Figure 3). The initial observations of peculiar crown galls on plants were made as early as 1679 (Malpighi, 1679). A significant advancement in understanding this phenomenon came in 1907, when it was confirmed that these plant tumors had a bacterial origin (Smith & Townsend, 1907). Further research led to the proposal of a tumor-inducing principle (Braun & Mandle, 1948; White & Braun, 1941). Subsequently, a large plasmid was

discovered in *A. tumefaciens*, which proved to have a crucial role in the bacterium's virulence (Van Larebeke *et al.*, 1975; Zaenen *et al.*, 1974). It was later discovered that *A. tumefaciens* has a unique mechanism of transferring a segment of its DNA, known as transfer DNA (T-DNA), into the host plant's genome. The oncogenes present on the T-DNA are expressed in plant cells, resulting in the production of plant hormones, which lead to uncontrolled cell division and tumor formation (Chilton *et al.*, 1977).

The process begins when *A. tumefaciens* detects phenolic compounds and sugars released by a wounded plant, which activate the virulence (Vir) genes on the tumor inducing (Ti) plasmid within the bacterium (Zupan & Zambryski, 1995). Several Vir genes encode proteins that form a type IV secretion system (T4SS), creating a channel between the bacterium and the plant cell. Other Vir genes encode proteins that process the T-DNA region of the Ti plasmid and guide it into the plant nucleus (Figure 4). The T-DNA, flanked by left and right border (LB and RB) sequences (Yadav *et al.*, 1982), is recognized by VirD1 and VirD2 proteins and released as a single-stranded molecule (Scheiffele *et al.*, 1995). VirD2 remains attached to the 5' end of the T-strand, guiding it during transfer. The T-strand, along with several virulence proteins, is then transferred into the plant cell through the T4SS (Ward *et al.*, 2002). Once inside the plant cell, the T-strand is coated with VirE2 proteins, which protect it from degradation and help guide it to the plant cell nucleus (Tzfira *et al.*, 2001). Once in the nucleus, the T-DNA is converted into double-stranded DNA, allowing for transient expression. Stable transformation occurs when the T-DNA integrates into the plant genome (Salomon & Puchta, 1998), a process facilitated by the host's own DSB repair machinery. However, the mechanism behind the integration process remained elusive for a long time.

The elucidation of the gene transfer mechanism between *Agrobacterium* and plants paved the way for the development of an efficient system for delivering genes into plant genomes (Joos *et al.*, 1983; Schell & Van Montagu, 1977). It was established that the oncogenes could be excised without impairing the bacterium's ability to insert DNA into the plant genome (Barton *et al.*, 1983; Bevan *et al.*, 1983; Framond *et al.*, 1983). The challenge posed by the large Ti plasmid was addressed by the introduction of binary plasmids, which separated the Vir genes and the T-DNA onto different vectors, while preserving the T-DNA transfer capability (Hoekema *et al.*, 1983).

T-DNA integration

After extensive research on the integration mechanism of T-DNA conducted by numerous laboratories, our lab's studies have conclusively demonstrated that the integration of T-DNA is critically dependent on Polθ. This enzyme mediates the error-prone repair process at the integration sites. This finding marks a significant advancement in our understanding of T-DNA integration. Using *A. thaliana* as a model, it was shown that mutants lacking Polθ were resistant to T-DNA integration, highlighting Polθ's essential role (van Kregten *et al.*, 2016). Later it was

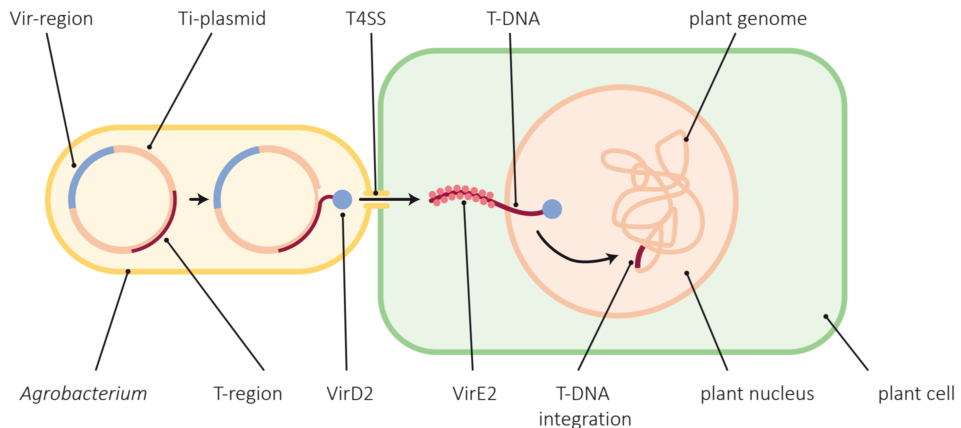


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Figure 4. Schematic representation of *Agrobacterium*-mediated transformation in plants. The transformation process begins with *Agrobacterium* recognizing plant wound sites and attaching to the plant cell. The T-DNA is excised from the Ti-plasmid by the VirD2 protein, which remains bound to the 5' end of the T-DNA. This T-DNA-VirD2 complex is then transferred, together with several other Vir proteins, to the plant cell through a type IV secretion system (T4SS). Inside the plant cell, the T-DNA is coated with VirE2 proteins to protect it from degradation. The T-DNA is subsequently transported to the plant nucleus, where it integrates into the plant genome using the host's double-strand break (DSB) repair machinery.

shown that the integration of the left border (LB) of the T-DNA is dependent on Polθ, while the right border (RB) can integrate via the two major pathways cNHEJ and TMEJ, after removal of covalently linked VirD2, mediated by tyrosyl phosphodiesterase 2 (TDP2) or the Mre11 endonuclease, respectively (Kralemann *et al.*, 2022).

T-DNA integrates at random positions in the genome, often resulting in genomic rearrangements including deletions, duplications, and translocations (Gang *et al.*, 2019; Hu *et al.*, 2017). The architecture of T-DNA insertions is often complex, with multiple T-DNA fragments integrating at a single site or across different genomic locations, which can have significant epigenomic impacts, influencing gene expression and stability (Jupe *et al.*, 2019). Additionally, chromosomal translocations are a common phenomenon in T-DNA insertion lines, resulting in the fusion of different chromosomal segments, potentially leading to the misregulation of genes at the breakpoints (Clark & Krysan, 2010). T-DNA integration can also cause a variety of other changes at the DNA level, such as the formation of new regulatory elements or the disruption of existing ones, leading to altered gene expression. The insertion process can be highly mutagenic, often disrupting coding sequences or regulatory regions, which is utilized in genetic studies to create mutant libraries for gene function analysis (Alonso *et al.*, 2003; Kleinboelting *et al.*, 2012). Furthermore, T-DNA integration can lead to significant epigenomic changes, such as alterations in DNA methylation and histone modifications, affecting gene expression and stability (Lieberman-Lazarovich *et al.*, 2013).

Gene targeting

In response to the challenges posed by random T-DNA integration, researchers have been exploring strategies for precise gene targeting. These methods aim to achieve error-free integration of T-DNA leveraging homologous recombination (HR) and a repair template carried by the T-DNA itself. Paszkowski and colleagues (1988) were the first to show that gene targeting is possible in plant cells. They demonstrated that a plasmid DNA, which included a segment of the kanamycin resistance gene, could be incorporated into the plant genome through homologous recombination (HR), albeit at a low frequency, allowing for the restoration of a functional gene. Furthermore, Offringa *et al.* (1990) expanded on this by showing that gene targeting could also be achieved in plant cells using *Agrobacterium*-mediated T-DNA transformation. Despite these advances, the frequency of gene targeting remained low, with about one successful event per 10^4 to 10^5 transformation events.

The introduction of DSBs in conjunction with gene targeting marked a significant advancement, particularly with the advent of CRISPR technology (Puchta and Fauser, 2013). This combination has greatly enhanced the efficiency of gene targeting, making it a more viable tool for plant genetic engineering. However, even in combination with DSB induction, gene targeting remains highly inefficient in plants (de Pater *et al*., 2009; 2013; 2018). Additionally, gene targeting in Polθ-deficient plants has been achieved (Kralemann *et al.*, 2024; Merker *et al.*, 2024; van Tol *et al.*, 2021), which abolishes random integration while retaining error-free gene targeting events. These studies demonstrate that Polθ is not essential for the gene targeting process itself. However, gene targeting often relies on the initial random integration of T-DNA, for which Polθ is required. These findings highlight the need for continued research and development to optimize these methods for routine use.

Aim of this thesis

Today, *Agrobacterium* is widely used as a vector to produce genetically modified plants. However, the randomness of T-DNA integration can complicate genetic modifications and lead to unpredictable results. Gene targeting, while offering error-free incorporation of a desired sequence on the T-DNA, suffers from inefficiency. In response to these challenges, there is a growing interest in devising methods for efficient, controlled and targeted T-DNA integration.

The CRISPR/Cas system emerges as a promising solution for targeted T-DNA integration by inducing a targeted DSB in which the T-DNA can integrate. The CRISPR reagents are introduced into the plant cell through the T-DNA itself via *Agrobacterium*-mediated transformation. This approach facilitates efficient integration of the T-DNA while reducing the risk of unintended mutations and ensuring more predictable transgene expression. However, further research is required to eliminate random integration and to resolve the issue of mutational end joining at the junctions.

Apart from facilitating targeted T-DNA integration, CRISPR/Cas can also be used for direct targeted mutagenesis. By targeting specific genomic sequences and facilitating repair through end joining, CRISPR/Cas can enable precisely located modifications. These modifications can range from gene knockouts to the introduction of specific mutations. All in all, leveraging CRISPR/Cas technology allows us to achieve greater accuracy in genetic modifications, paving the way for more reliable and stable outcomes in plant biotechnology.

Currently, there is limited understanding of the repair mechanisms for CRISPR/Cas-induced DSBs in higher plants, and their relationship to T-DNA integration at these sites. To address this, we conducted a high-resolution mutational analysis of DSB repair and targeted T-DNA integration sites. Additionally, we developed and explored alternative techniques to facilitate targeted and predictable gene editing in plants. This thesis aims to elucidate the pathways governing these mechanisms and provide insights into their parameters, which is crucial for improving the precision and efficiency of CRISPR-based genome editing technologies.

Outline of this thesis

In **Chapter 2**, we investigated the mechanisms of DSB repair in plants, focusing on the roles of classical nonhomologous end joining (cNHEJ) and polymerase theta-mediated end joining (TMEJ). Our study provides a comprehensive analysis of how these end joining pathways contribute to the repair of CRISPR/Cas9-induced DSBs in *A. thaliana*. Additionally, we examined the targeted capture of T-DNA within these induced breaks.

In **Chapter 3**, we systematically compared the DNA repair outcomes and pathways activated by Cas9 and Cas12a in *A. thaliana*. We provide detailed repair profiles for both nucleases, showing that DSB-end configuration does not dictate repair pathway choice. The most notable disparity between Cas9 and Cas12a is the formation of 1 bp insertions, which we here show to depend on polymerase Lambda. This chapter also highlights that Cas12a, like Cas9, stimulates the integration of T-DNA molecules.

In **Chapter 4**, to find a more precise and predictable manner of DSB repair, we analyzed repair of genomic sites in which the DSB was flanked by short direct repeats (extended microhomology). We surprisingly found that eMMEJ, which was the prevalent outcome in wild-type plants, was absent in a Polθ deficient mutant. In "standard" sequence contexts the polymerase activity of Polθ is required to kick-start DNA synthesis using micro-homologous pairing of DSB ends. Intriguingly, we discovered that DSBs containing eMH rely solely on the helicase activity of Polθ, rendering the polymerase domain obsolete.

In **Chapter 5**, we studied DSB induction both on T-DNA and within the genome. Our findings reveal that the repair process for DSBs on T-DNA reflects that of DSBs in the genome, resulting in similar scars at the repaired sites. Additionally, we demonstrated that T-DNA can be cleaved with nearly 100% efficiency before being captured by the genomic target site, enabling targeted capture of T-DNA without unwanted sequences. Furthermore, we showed that upon simultaneous DSB induction at two sites, the ends of the breaks frequently fuse together, leading to genomic aberrations.

In **Chapter 6**, we used the knowledge obtained in the previous chapters to design a novel method to facilitate precise targeted modification of the plant genome using T-DNA containing eMH. We found that eMH-mediated capture presented a major fraction of all T-DNA capture events, enabling targeted and error-free integration of genes of interest. Additionally, we showed that more subtle mutations can be achieved by using a dual T-DNA approach: a helper-T-DNA facilitates targeted DSB induction, whereas a smaller T-DNA provides an eMH-based repair template to insert the desired mutation. In conclusion, this study effectively demonstrates a novel approach for targeted genome modifications, which notably surpasses the efficiency of traditional gene targeting methods.

References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., . . . Gadrinab, C. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science, 301(5633), 653-657.

Anand, R., Buechelmaier, E., Belan, O., Newton, M., Vancevska, A., Kaczmarczyk, A., . . . Boulton, S. J. (2022). HELQ is a dual-function DSB repair enzyme modulated by RPA and RAD51. Nature, 601(7892), 268-273. doi:10.1038/s41586-021-04261-0

Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature, 408(6814), 796-815.

Badhan, S., Ball, A. S., & Mantri, N. (2021). First Report of CRISPR/Cas9 Mediated DNA-Free Editing of 4CL and RVE7 Genes in Chickpea Protoplasts. Int J Mol Sci, 22(1). doi:10.3390/ijms22010396

Bari, V. K., Nassar, J. A., & Aly, R. (2021). CRISPR/Cas9 mediated mutagenesis of MORE AXILLARY GROWTH 1 in tomato confers resistance to root parasitic weed Phelipanche aegyptiaca. Sci Rep, 11(1), 3905. doi:10.1038/s41598-021-82897-8

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., . . . Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science, 315(5819), 1709-1712. doi:10.1126/science.1138140

Barton, K. A., Binns, A. N., Matzke, A. J., & Chilton, M. D. (1983). Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell, 32(4), 1033-1043. doi:10.1016/0092-8674(83)90288-x

Beagan, K., Armstrong, R. L., Witsell, A., Roy, U., Renedo, N., Baker, A. E., . . . McVey, M. (2017). Drosophila DNA polymerase theta utilizes both helicase-like and polymerase domains during microhomology-mediated end joining and interstrand crosslink repair. PLoS Genet, 13(5), e1006813. doi:10.1371/journal.pgen.1006813

Betermier, M., Bertrand, P., & Lopez, B. S. (2014). Is non-homologous end-joining really an inherently error-prone process? PLoS Genet, 10(1), e1004086. doi:10.1371/journal.pgen.1004086

Bevan, M. W., Flavell, R. B., & Chilton, M.-D. (1983). A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature, 304(5922), 184-187. doi:10.1038/304184a0

Bhargava, R., Onyango, D. O., & Stark, J. M. (2016). Regulation of Single-Strand Annealing and its Role in Genome Maintenance. Trends Genet, 32(9), 566-575. doi:10.1016/j.tig.2016.06.007

Blanvillain-Baufume, S., Reschke, M., Sole, M., Auguy, F., Doucoure, H., Szurek, B., . . . Koebnik, R. (2017). Targeted promoter editing for rice resistance to Xanthomonas oryzae pv. oryzae reveals differential activities for SWEET14-inducing TAL effectors. Plant Biotechnol J, 15(3), 306-317. doi:10.1111/pbi.12613

Braun, A., & Mandle, R. (1948). Studies on the inactivation of the tumor-inducing principle in crown gall. Growth, 12, 255–269.

Callaway, E. (2018). CRISPR plants now subject to tough GM laws in European Union. Nature, 560(7716), 16-17.

Chang, H. H. Y., Pannunzio, N. R., Adachi, N., & Lieber, M. R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nat Rev Mol Cell Biol, 18(8), 495-506. doi:10.1038/nrm.2017.48

Chilton, M.-D., Drummond, M. H., Merlo, D. J., Sciaky, D., Montoya, A. L., Gordon, M. P., & Nester, E. W. (1977). Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell, 11(2), 263-271.

Clark, K. A., & Krysan, P. J. (2010). Chromosomal translocations are a common phenomenon in *Arabidopsis thaliana* T-DNA insertion lines. Plant J, 64(6), 990-1001. doi:10.1111/j.1365-313X.2010.04386.x

de Pater, S., Klemann, B. J. P. M., & Hooykaas, P. J. J. (2018). True gene-targeting events by CRISPR/Cas-induced DSB repair of the PPO locus with an ectopically integrated repair template. Sci Rep, 8(1), 3338. doi:10.1038/s41598-018-21697-z

de Pater, S., Neu*teb*oom, L. W., Pinas, J. E., Hooykaas, P. J. J., & van der Zaal, B. J. (2009). ZFN-induced mutagenesis and gene-targeting in *Arabidopsis* through *Agrobacterium*-mediated floral dip transformation. Plant Biotechnol J, 7(8), 821-835. doi:10.1111/j.1467-7652.2009.00446.x

de Pater, S., Pinas, J. E., Hooykaas, P. J. J., & van der Zaal, B. J. (2013). ZFN-mediated gene targeting of the *Arabidopsis* protoporphyrinogen oxidase gene through *Agrobacterium*-mediated floral dip transformation. Plant Biotechnol J, 11(4), 510-515. doi:10.1111/pbi.12040

Deriano, L., & Roth, D. B. (2013). Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. Annu Rev Genet, 47, 433-455. doi:10.1146/annurev-genet-110711-155540

Doutriaux, M. P., Couteau, F., Bergounioux, C., & White, C. (1998). Isolation and characterisation of the RAD51 and DMC1 homologs from *Arabidopsis thaliana*. Molecular and General Genetics MGG, 257(3), 283-291. doi:10.1007/s004380050649

Du, Y. T., Zhao, M. J., Wang, C. T., Gao, Y., Wang, Y. X., Liu, Y. W., . . . Ma, Y. Z. (2018). Identification and characterization of GmMYB118 responses to drought and salt stress. BMC Plant Biol, 18(1), 320. doi:10.1186/s12870-018-1551-7

Elbakry, A., & Lobrich, M. (2021). Homologous Recombination Subpathways: A Tangle to Resolve. Front Genet, 12, 723847. doi:10.3389/fgene.2021.723847

Eschbach, V., & Kobbe, D. (2014). Different replication protein A complexes of *Arabidopsis thaliana* have different DNA-binding properties as a function of heterotrimer composition. Plant Cell Physiol, 55(8), 1460-1472. doi:10.1093/pcp/pcu076

European Commission. (2018). Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32001L0018

European Commission. (2021). Study on the status of new genomic techniques under Union law and in light of the Court of Justice ruling in Case C-528/16. Retrieved from https://food.ec.europa.eu/plants/genetically-modified-organisms/new-techniques-biotechnology/ec-study-new-genomic-techniques_en

Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D. L., Wei, P., . . . Zhu, J. K. (2013). Efficient genome editing in plants using a CRISPR/Cas system. Cell Res, 23(10), 1229-1232. doi:10.1038/cr.2013.114

Framond, A. J. d., Barton, K. A., & Chilton, M. D. J. B. T. (1983). Mini–Ti: A New Vector Strategy for Plant Genetic Engineering. 1, 262-269.

Gang, H., Liu, G., Zhang, M., Zhao, Y., Jiang, J., & Chen, S. J. B. g. (2019). Comprehensive characterization of T-DNA integration induced chromosomal rearrangement in a birch T-DNA mutant. 20, 1-11.

Haverkort, A. J., Boonekamp, P. M., Hutten, R., Jacobsen, E., Lotz, L. A. P., Kessel, G. J. T., . . . Visser, R. G. F. (2016). Durable Late Blight Resistance in Potato Through Dynamic Varieties Obtained by Cisgenesis: Scientific and Societal Advances in the DuRPh Project. Potato Research, 59(1), 35-66. doi:10.1007/s11540-015-9312-6

He, P., & Yang, W. (2018). Template and primer requirements for DNA Pol theta-mediated end joining. Proc Natl Acad Sci U S A, 115(30), 7747-7752. doi:10.1073/pnas.1807329115

Hoekema, A., Hirsch, P. R., Hooykaas, P. J., & Schilperoort, R. A. (1983). A binary plant vector strategy based on separation of vir-and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature, 303(5913), 179-180.

Hu, Y., Chen, Z., Zhuang, C., & Huang, J. (2017). Cascade of chromosomal rearrangements caused by a heterogeneous T-DNA integration supports the double-stranded break repair model for T-DNA integration. Plant J, 90(5), 954-965. doi:10.1111/tpj.13523

Inagaki, S., Suzuki, T., Ohto, M. A., Urawa, H., Horiuchi, T., Nakamura, K., & Morikami, A. (2006). *Arabidopsis* TEBICHI, with helicase and DNA polymerase domains, is required for regulated cell division and differentiation in meristems. The Plant Cell, 18(4), 879-892. doi:10.1105/tpc.105.036798

Ishibashi, T., Kimura, S., Furukawa, T., Hatanaka, M., Hashimoto, J., & Sakaguchi, K. (2001). Two types of replication protein A 70 kDa subunit in rice, Oryza sativa: molecular cloning, characterization, and cellular & tissue distribution. Gene, 272(1), 335-343. doi:https://doi.org/10.1016/S0378-1119(01)00555-8

Jackson, S. P. (2002). Sensing and repairing DNA double-strand breaks. Carcinogenesis, 23(5), 687-696.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. J. s. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. 337(6096), 816-821.

Jones, H. D. (2015). Regulatory uncertainty over genome editing. Nat Plants, 1, 14011. doi:10.1038/nplants.2014.11

Joos, H., Timmerman, B., Van Montagu, M., & Schell, J. (1983). Genetic analysis of transfer and stabilization of *Agrobacterium* DNA in plant cells. The EMBO Journal, 2(12), 2151-2160. doi:10.1002/j.1460-2075.1983.tb01716.x

Jouanin, A., Schaart, J. G., Boyd, L. A., Cockram, J., Leigh, F. J., Bates, R., . . . Smulders, M. J. M. (2019). Outlook for coeliac disease patients: towards bread wheat with hypoimmunogenic gluten by gene editing of alpha- and gamma-gliadin gene families. BMC Plant Biol, 19(1), 333. doi:10.1186/s12870-019-1889-5

Jupe, F., Rivkin, A. C., Michael, T. P., Zander, M., Motley, S. T., Sandoval, J. P., . . . Ecker, J. R. (2019). The complex architecture and epigenomic impact of plant T-DNA insertions. PLoS Genet, 15(1), e1007819. doi:10.1371/journal.pgen.1007819

Kamp, J. A., Lemmens, B. B. L. G., Romeijn, R. J., Changoer, S. C., van Schendel, R., & Tijsterman, M. (2021). Helicase Q promotes homology-driven DNA double-strand break repair and prevents tandem duplications. Nature Communications, 12(1). doi:10.1038/s41467-021-27408-z

Kelso, A. A., Lopezcolorado, F. W., Bhargava, R., & Stark, J. M. (2019). Distinct roles of RAD52 and POLQ in chromosomal break repair and replication stress response. PLoS Genet, 15(8), e1008319. doi:10.1371/journal.pgen.1008319

Kent, T., Chandramouly, G., McDevitt, S. M., Ozdemir, A. Y., & Pomerantz, R. T. (2015). Mechanism of microhomology-mediated end-joining promoted by human DNA polymerase theta. Nat Struct Mol Biol, 22(3), 230-237. doi:10.1038/nsmb.2961

Klap, C., Yeshayahou, E., Bolger, A. M., Arazi, T., Gupta, S. K., Shabtai, S., . . . Barg, R. (2017). Tomato facultative parthenocarpy results from SlAGAMOUS-LIKE 6 loss of function. Plant Biotechnol J, 15(5), 634-647. doi:10.1111/pbi.12662

Kleinboelting, N., Huep, G., Kloetgen, A., Viehoever, P., & Weisshaar, B. J. N. a. r. (2012). GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. 40(D1), D1211-D1215.

Koornneef, M., & Meinke, D. (2010). The development of *Arabidopsis* as a model plant. The Plant Journal, 61(6), 909-921.

Kralemann, L. E. M., de Pater, S., Shen, H., Kloet, S. L., van Schendel, R., Hooykaas, P. J. J., & Tijsterman, M. (2022). Distinct mechanisms for genomic attachment of the 5' and 3' ends of *Agrobacterium* T-DNA in plants. Nat Plants, 8(5), 526-534. doi:10.1038/s41477-022-01147-5

Kralemann, L. E. M., van Tol, N., Hooykaas, P. J. J., & Tijsterman, M. (2024). Molecular analysis of the role of polymerase theta in gene targeting in *Arabidopsis thaliana*. Plant J. doi:10.1111/tpj.16689

Kumar, M., Prusty, M. R., Pandey, M. K., Singh, P. K., Bohra, A., Guo, B., & Varshney, R. K. (2023). Application of CRISPR/Cas9-mediated gene editing for abiotic stress management in crop plants. Front Plant Sci, 14, 1157678. doi:10.3389/fpls.2023.1157678

Lawrence, K. S., Chau, T., & Engebrecht, J. (2015). DNA damage response and spindle assembly checkpoint function throughout the cell cycle to ensure genomic integrity. PLoS Genet, 11(4), e1005150. doi:10.1371/journal.pgen.1005150

Li, J.-F., Norville, J. E., Aach, J., McCormack, M., Zhang, D., Bush, J., . . . Sheen, J. (2013). Multiplex and homologous recombination–mediated genome editing in *Arabidopsis* and Nicotiana benthamiana using guide RNA and Cas9. Nature Biotechnology, 31(8), 688-691. doi:10.1038/nbt.2654

Lieberman-Lazarovich, M., Melamed-Bessudo, C., de Pater, S., & Levy, A. A. (2013). Epigenetic alterations at genomic loci modified by gene targeting in *Arabidopsis thaliana*. PLoS One, 8(12), e85383. doi:10.1371/journal.pone.0085383

Lou, D., Wang, H., Liang, G., & Yu, D. (2017). OsSAPK2 Confers Abscisic Acid Sensitivity and Tolerance to Drought Stress in Rice. Front Plant Sci, 8, 993. doi:10.3389/fpls.2017.00993

Malpighi, M. (1679). On galls. Anotomia Plantarum.

Marini, F., & Wood, R. D. (2002). A human DNA helicase homologous to the DNA cross-link sensitivity protein Mus308. J Biol Chem, 277(10), 8716-8723. doi:10.1074/jbc.M110271200

Mateos-Gomez, P. A., Kent, T., Deng, S. K., McDevitt, S., Kashkina, E., Hoang, T. M., . . . Sfeir, A. (2017). The helicase domain of Polθ counteracts RPA to promote alt-NHEJ. Nature Structural & Molecular Biology, 24(12), 1116 - 1128.

Merker, L., Feller, L., Dorn, A., & Puchta, H. (2024). 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*. Plant J. doi:10.1111/tpj.16604

Mimitou, E. P., & Symington, L. S. (2009). DNA end resection: many nucleases make light work. DNA Repair (Amst), 8(9), 983-995. doi:10.1016/j.dnarep.2009.04.017

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D. G., & Kamoun, S. (2013). Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nature Biotechnology, 31(8), 691-693. doi:10.1038/nbt.2655

Newman, J. A., Cooper, C. D. O., Aitkenhead, H., & Gileadi, O. (2015). Structure of the Helicase Domain of DNA Polymerase Theta Reveals a Possible Role in the Microhomology-Mediated End-Joining Pathway. Structure, 23(12), 2319-2330. doi:10.1016/j.str.2015.10.014

Oliva, R., Ji, C., Atienza-Grande, G., Huguet-Tapia, J. C., Perez-Quintero, A., Li, T., . . . Yang, B. (2019). Broad-spectrum resistance to bacterial blight in rice using genome editing. Nat Biotechnol, 37(11), 1344-1350. doi:10.1038/s41587-019-0267-z

Ortigosa, A., Gimenez-Ibanez, S., Leonhardt, N., & Solano, R. (2019). Design of a bacterial speck resistant tomato by CRISPR/Cas9-mediated editing of SlJAZ2. Plant Biotechnol J, 17(3), 665-673. doi:10.1111/pbi.13006

Ozdemir, A. Y., Rusanov, T., Kent, T., Siddique, L. A., & Pomerantz, R. T. (2018). Polymerase theta-helicase efficiently unwinds DNA and RNA-DNA hybrids. J Biol Chem, 293(14), 5259-5269. doi:10.1074/jbc.RA117.000565

Qiu, Z., Kang, S., He, L., Zhao, J., Zhang, S., Hu, J., . . . Zhu, L. (2018). The newly identified heat-stress sensitive albino 1 gene affects chloroplast development in rice. Plant Sci, 267, 168-179. doi:10.1016/j.plantsci.2017.11.015

Puchta, H., & Fauser, F. (2013). Gene targeting in plants: 25 years later. International Journal of Developmental Biology, 57(6-7-8), 629-637.

Ramsden, D. A., Carvajal-Garcia, J., & Gupta, G. P. (2022). Mechanism, cellular functions and cancer roles of polymerase-theta-mediated DNA end joining. Nat Rev Mol Cell Biol, 23(2), 125-140. doi:10.1038/s41580-021-00405-2

Samach, A., Melamed-Bessudo, C., Avivi-Ragolski, N., Pietrokovski, S., & Levy, A. A. (2011). Identification of plant RAD52 homologs and characterization of the *Arabidopsis thaliana* RAD52-like genes. Plant Cell, 23(12), 4266-4279. doi:10.1105/tpc.111.091744

Sanchez-Leon, S., Gil-Humanes, J., Ozuna, C. V., Gimenez, M. J., Sousa, C., Voytas, D. F., & Barro, F. (2018). Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol J, 16(4), 902-910. doi:10.1111/pbi.12837

Santillan Martinez, M. I., Bracuto, V., Koseoglou, E., Appiano, M., Jacobsen, E., Visser, R. G. F., . . . Bai, Y. (2020). CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene PMR4 for resistance against powdery mildew. BMC Plant Biol, 20(1), 284. doi:10.1186/s12870-020-02497-y

Schaub, J. M., Soniat, M. M., & Finkelstein, I. J. (2022). Polymerase theta-helicase promotes end joining by stripping single-stranded DNA-binding proteins and bridging DNA ends. Nucleic Acids Res, 50(7), 3911-3921. doi:10.1093/nar/gkac119

Scheiffele, P., Pansegrau, W., & Lanka, E. (1995). Initiation of *Agrobacterium tumefaciens* T-DNA processing. Purified proteins VirD1 and VirD2 catalyze site- and strand-specific cleavage of superhelical T-border DNA in vitro. J Biol Chem, 270(3), 1269-1276. doi:10.1074/jbc.270.3.1269

Schell, J., & Van Montagu, M. (1977). The Ti-Plasmid of *Agrobacterium*Tumefaciens, A Natural Vector for the Introduction of NIF Genes in Plants? In A. Hollaender, R. H. Burris, P. R. Day, R. W. F. Hardy, D. R. Helinski, M. R.

Lamborg, L. Owens, & R. C. Valentine (Eds.), Genetic Engineering for Nitrogen Fixation (pp. 159-179). Boston, MA: Springer US.

Schimmel, J., van Schendel, R., den Dunnen, J. T., & Tijsterman, M. (2019). Templated Insertions: A Smoking Gun for Polymerase Theta-Mediated End Joining. Trends Genet, 35(9), 632-644. doi:10.1016/j.tig.2019.06.001

Seki, M., Marini, F., & Wood, R. D. (2003). POLQ (Pol theta), a DNA polymerase and DNA-dependent ATPase in human cells. Nucleic Acids Res, 31(21), 6117-6126. doi:10.1093/nar/gkg814

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., . . . Gao, C. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. Nature Biotechnology, 31(8), 686-688. doi:10.1038/nbt.2650

Shi, J., Gao, H., Wang, H., Lafitte, H. R., Archibald, R. L., Yang, M., . . . Habben, J. E. (2017). ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol J, 15(2), 207-216. doi:10.1111/pbi.12603

Smith, E., & Townsend, C. (1907). A plant-tumor of bacterial origin. Science, 25, 239-241.

Swartjes, T., Staals, R. H. J., & van der Oost, J. (2020). Editor's cut: DNA cleavage by CRISPR RNA-guided nucleases Cas9 and Cas12a. Biochem Soc Trans, 48(1), 207-219. doi:10.1042/BST20190563

Symington, L. S., Rothstein, R., & Lisby, M. (2014). Mechanisms and regulation of mitotic recombination in Saccharomyces cerevisiae. Genetics, 198(3), 795-835. doi:10.1534/genetics.114.166140

Tamura, K., Adachi, Y., Chiba, K., Oguchi, K., & Takahashi, H. (2001). Identification of Ku70 and Ku80 homologues in *Arabidopsis thaliana*: evidence for a role in the repair of DNA double-strand breaks. The Plant Journal, 29(6), 771-781.

The Royal Swedish Academy of Sciences. (2020). Press release: The Nobel Prize in Chemistry 2020. Retrieved from https://www.nobelprize.org/prizes/chemistry/2020/press-release/

Tzfira, T., Vaidya, M., & Citovsky, V. (2001). VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. The EMBO Journal.

van Attikum, H., Bundock, P., Overmeer, R. M., Lee, L. Y., Gelvin, S. B., & Hooykaas, P. J. J. (2003). The *Arabidopsis* AtLIG4 gene is required for the repair of DNA damage, but not for the integration of *Agrobacterium* T-DNA. Nucleic acids research, 31(14), 4247-4255. doi:10.1093/nar/gkg458

Van Gent, D. C., Hoeijmakers, J. H., & Kanaar, R. (2001). Chromosomal stability and the DNA double-stranded break connection. Nature Reviews Genetics, 2(3), 196-206.

van Kregten, M., de Pater, S., Romeijn, R., van Schendel, R., Hooykaas, P. J. J., & Tijsterman, M. (2016). T-DNA integration in plants results from polymerase-theta-mediated DNA repair. Nat Plants, 2(11), 16164. doi:10.1038/nplants.2016.164

Van Larebeke, N., Genetello, C. H., Schell, J., Schilperoort, R. A., Hermans, A. K., Hernalsteens, J. P., & Van Montagu, M. (1975). Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature, 255(5511), 742-743. doi:10.1038/255742a0

van Tol, N., van Schendel, R., Bos, A., van Kregten, M., de Pater, S., Hooykaas, P. J. J., & Tijsterman, M. (2021). Gene targeting in polymerase theta-deficient *Arabidopsis thaliana*. Plant J. doi:10.1111/tpj.15557

Vanson, S., Li, Y., Wood, R. D., & Doublie, S. (2022). Probing the structure and function of polymerase theta helicase-like domain. DNA Repair (Amst), 116, 103358. doi:10.1016/j.dnarep.2022.103358

Wang, X., & Haber, J. E. (2004). Role of Saccharomyces single-stranded DNA-binding protein RPA in the strand invasion step of double-strand break repair. PLoS Biol, 2(1), E21. doi:10.1371/journal.pbio.0020021

Ward, D. V., Draper, O., Zupan, J. R., & Zambryski, P. C. (2002). Peptide linkage mapping of the *Agrobacterium tumefaciens* vir-encoded type IV secretion system reveals protein subassemblies. Proceedings of the National Academy of Sciences, 99(17), 11493-11500.

White, P., & Braun, A. (1941). Crown gall production by bacteria-free tumor tissues. Science, 223, 1175–1176.

Wood, R. D., & Doublie, S. (2016). DNA polymerase theta (POLQ), double-strand break repair, and cancer. DNA Repair (Amst), 44, 22-32. doi:10.1016/j.dnarep.2016.05.003

Wyatt, D. W., Feng, W., Conlin, M. P., Yousefzadeh, M. J., Roberts, S. A., Mieczkowski, P., . . . Ramsden, D. A. (2016). Essential Roles for Polymerase theta-Mediated End Joining in the Repair of Chromosome Breaks. Mol Cell, 63(4), 662-673. doi:10.1016/j.molcel.2016.06.020

Xie, K., & Yang, Y. (2013). RNA-guided genome editing in plants using a CRISPR-Cas system. Mol Plant, 6(6), 1975-1983. doi:10.1093/mp/sst119

Yadav, N. S., Vanderleyden, J., Bennett, D. R., Barnes, W. M., & Chilton, M.-D. (1982). Short direct repeats flank the T-DNA on a nopaline Ti plasmid. Proceedings of the National Academy of Sciences, 79(20), 6322-6326.

Yousefzadeh, M. J., & Wood, R. D. (2013). DNA polymerase POLQ and cellular defense against DNA damage. DNA Repair (Amst), 12(1), 1-9. doi:10.1016/j.dnarep.2012.10.004

Yuan, M., Zhu, J., Gong, L., He, L., Lee, C., Han, S., . . . He, G. (2019). Mutagenesis of FAD2 genes in peanut with CRISPR/Cas9 based gene editing. BMC Biotechnol, 19(1), 24. doi:10.1186/s12896-019-0516-8

Zaenen, I., Van Larebeke, N., Van Montagu, M., & Schell, J. (1974). Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. J Mol Biol, 86(1), 109-127. doi:10.1016/s0022-2836(74)80011-2

Zsögön, A., Cermak, T., Naves, E. R., Notini, M. M., Edel, K. H., Weinl, S., . . . Peres, L. E. P. (2018). De novo domestication of wild tomato using genome editing. Nat Biotechnol. doi:10.1038/nbt.4272

Zupan, J. R., & Zambryski, P. (1995). Transfer of T-DNA from *Agrobacterium* to the plant cell. Plant Physiology, 107(4), 1041.