



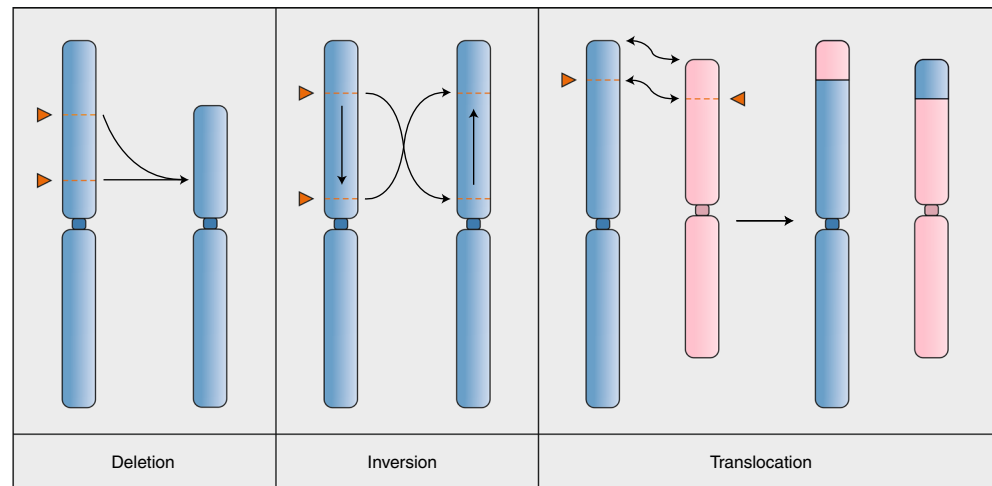
# CRISPR–Cas9-mediated chromosome engineering in *Arabidopsis thaliana*

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The rise of the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas) system has made it possible to induce double-strand breaks at almost any desired target site in the genome. In plant somatic cells, double-strand breaks are predominantly repaired by the error-prone nonhomologous end-joining pathway, which can lead to mutations at the break site upon repair. So far, it had only been possible to induce genomic changes of up to a few hundred kilobases in plants utilizing this mechanism. However, by combining the highly efficient *Staphylococcus aureus* Cas9 (SaCas9) with an egg-cell-specific promoter to facilitate heritable mutations, chromosomal rearrangements in the Mb range, such as inversion and translocations, were obtained in *Arabidopsis thaliana* recently. Here we describe the chromosome-engineering protocol used to generate these heritable chromosomal rearrangements in *A. thaliana*. The protocol is based on *Agrobacterium*-mediated transformation of *A. thaliana* with transfer DNA constructs containing SaCas9, which is driven by an egg-cell-specific promoter, and two guide RNAs that have been preselected based on their cutting efficiency. In the T1 generation, primary transformants are selected and, if required, analyzed by Droplet Digital PCR and propagated. In the following generations, junction-specific PCR screenings are carried out until plants that carry the rearrangement homozygously are identified. Using this protocol, overall rearrangement frequencies range between 0.03% and 0.5%, depending on the type of rearrangement. In total, it takes about 1 year to establish homozygous lines.

## Introduction

The emergence and wide applicability of the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas) system has allowed plant biologists to induce genomic changes in an unprecedented way. Having started with small-scale changes, such as the induction of double-strand breaks (DSBs) within trait-related genes to improve quantitative and qualitative characteristics, it is now feasible to restructure entire plant chromosomes, which takes the plant breeding process to a completely new level<sup>1,2</sup>. If two DSBs are induced simultaneously on the same or on different chromosomes, chromosomal rearrangements, such as inversions and translocations, can be induced (Fig. 1). In plants, chromosomal rearrangements strongly contribute to evolutionary processes and are prevalent in a variety of plant families<sup>3</sup>. While small-scale chromosomal rearrangements on the kbp level had already been successfully induced in plants, the induction of large-scale chromosomal rearrangements had not been described until recently<sup>4–9</sup>. However, they are of tremendous interest for plant breeders as they provide the means for the targeted manipulation of genetic linkages, by breaking as well as stabilizing genetic linkages between traits, and for the manipulation of the recombination landscape, the foundation of genetic exchange<sup>2</sup>. Just recently, we and others reported CRISPR–Cas9-mediated large-scale chromosomal rearrangements on the Mbp level in the model plant *Arabidopsis* and the crop plant maize<sup>5,6,10</sup>. Here, we describe the protocol to generate large-scale chromosomal rearrangements in *Arabidopsis thaliana* (Fig. 2). For the generation of inversions and translocations, two DSBs need to be induced simultaneously. As high cleavage efficiencies are a prerequisite, mutation rates at potential target sites are evaluated before the start of the actual experiment (stage 1, Steps 1–81). Optionally, the overall chromosome-engineering efficiency can be tested by Droplet Digital PCR (ddPCR) analysis (stage 1, Steps 82–111). To induce the respective rearrangements, the plants are transformed with a transfer DNA (T-DNA) comprising Cas9 from *Staphylococcus aureus* (SaCas9) under control of an egg-cell-specific promoter and the two respective guide RNAs (gRNAs). Transgenic plants are selected and propagated (stage 2, Steps 112–117). In the T2 generation, the T1 lines are subjected to bulk DNA extraction and jointly

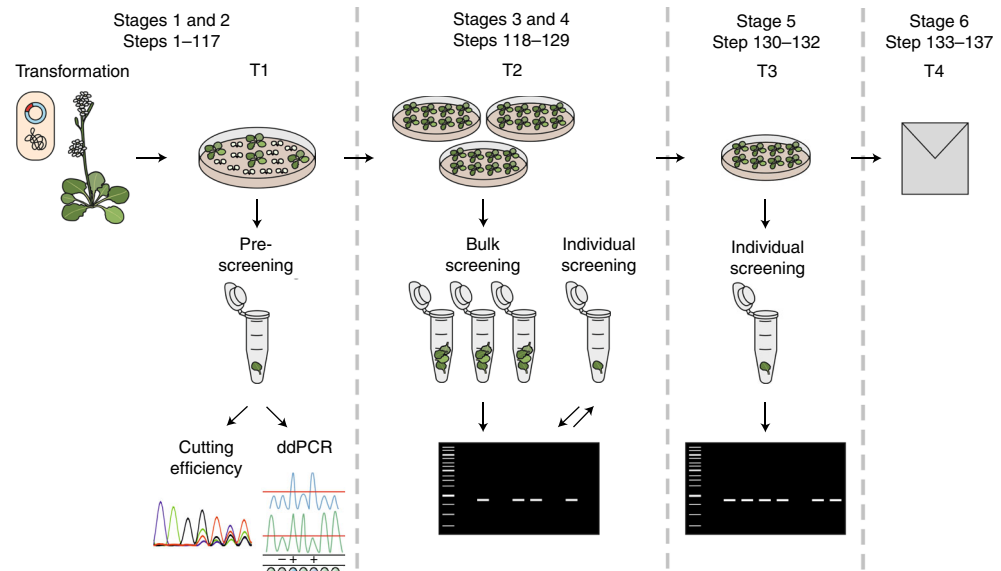


**Fig. 1 | Examples of chromosomal rearrangements generated by chromosome engineering.** Orange arrowheads indicate CRISPR-Cas-induced DSBs.

screened for the chromosomal rearrangement by PCR using primers specific to the newly formed junctions. In the case of positive results, the single plants of the pooled T1 lines are subjected to individual DNA extraction and screened individually again (stages 3 and 4, Steps 118–129). In the T3 generation, the chromosomal rearrangement is confirmed in the offspring (stage 5, Steps 130–132), which is then propagated to acquire enough seed material (stage 6, Step 133–137). In this way, plants homozygous for the respective chromosomal rearrangements can be identified in a medium- to high-throughput procedure.

### Development of the protocol

CRISPR-Cas-mediated mutagenesis in plants is now an everyday occurrence in most laboratories with standardized protocols, requiring very little effort and consideration. However, it is only recently that the targeted induction of chromosomal rearrangements has been achieved, as it was previously hampered by the limitations of former genome editing tools and the lack of heritable events<sup>11</sup>. In contrast to common nonhomologous end joining (NHEJ)-mediated mutations, chromosomal rearrangements occur less frequently and, thus, demand considerably more groundwork. Recent data from experiments in *Arabidopsis* revealed that chromosomal rearrangements induced by current CRISPR technologies occur at very low frequencies, indicating that the induced DSBs are only rarely converted into chromosomal rearrangements<sup>6</sup>. Therefore, it is necessary to establish a convenient screening protocol to identify such rare events. Other sophisticated changes, such as those induced by gene targeting (GT), allow identification by stable selection markers. However, a similar selection process is not applicable in the case of chromosomal rearrangements<sup>12</sup>. Moreover, to make the protocol applicable for breeding purposes, it is highly desirable to bypass the need for any kind of stably integrated transgene that would necessitate elaborate outcrossing. The same is true for the application of GT in plant breeding. Therefore, we and others already proposed and established a selection-marker-free PCR screening protocol that facilitates the selection of positive GT plants through a hierarchically structured screening from bulk to individual plant<sup>13–15</sup>. In this way, large amounts of plant material can be analyzed simultaneously in pools and screening of individual plants only becomes necessary if a positive signal is detected within the pool. When this bulk screening protocol turned out to be very successful in the case of GT, we adapted the same protocol for the selection of chromosomal rearrangements. Here, we could show that the bulk screening protocol is also successfully applicable to the detection of inversions in the kbp, as well as Mbp, range, and also to the detection of reciprocal translocations, even though the frequency of chromosomal rearrangement events is orders of magnitude lower than that of GT events<sup>4–6</sup>. The major advantage of this screening system is that the adaptation to different chromosomal changes is only mediated through unique junction-specific primer combinations. In addition, we observed a correlation between inversion frequencies and mutation rates as a proxy for cutting efficiencies at the respective target sites,



**Fig. 2 | Screening strategy for the identification of chromosomal rearrangements and establishment of homozygous lines.** Transformation (stages 1 and 2). Ten to twenty *A. thaliana* plants are transformed by floral dip transformation with the T-DNA construct containing one (for determination of the mutation frequency) or two (for the induction of chromosomal rearrangements) gRNA cassettes. The plants are cultivated until seed set. T1 (stages 1 and 2): primary transformants are selected on herbicide-containing GM medium. For subsequent PCR screenings in the T2 generation, 40–80 plants are propagated. Optionally, the DNA of 100 primary transformants is extracted for ddPCR analysis, which can be used to determine the overall genome-engineering efficiency. For determination of the cutting efficiency at the target site, the DNA of ten primary transformants is extracted, the cut site amplified by PCR and the amplicons sequenced. The sequencing results are analyzed using the TIDE tool. T2 (stages 3 and 4): starting in the T2 generation, chromosomal rearrangements are identified by PCR screenings using junction-specific primers. First, the DNA of 40 plants per line is pooled and screened jointly. Only in the case of a positive result, the plants of the pool are individually screened again. The newly formed junctions are sequenced and the positive plants are propagated. T3 (stage 5): in the T3 generation, plants are additionally genotyped to identify homozygous lines and screened for the presence of the T-DNA construct. Preferably, only homozygous plants are propagated that do not carry the T-DNA construct anymore. T4 (stage 6): the resulting T4 seeds are homozygous.

indicating that DSB induction at the target sites is the rate-limiting step for the induction of chromosomal rearrangements. Therefore, a critical addition to this screening process is that cleavage efficiencies at the target sites need to be analyzed before the start of the experiment. This is necessary to ensure an optimal cutting efficiency concerning the, already infrequent, occurrence of chromosomal rearrangements. This applies to all of the target sites as no fewer than two target sites are required to induce chromosomal rearrangements. Also, we used the Cas9 ortholog of *S. aureus*, which showed superior editing in *A. thaliana*, and an egg-cell-specific promoter that promotes germline transmission to induce chromosomal rearrangements. By combining all those steps, we established this protocol that allowed us to induce the first described Mbp-sized inversion and reciprocal translocations in plants<sup>5,6</sup>.

### Applications

This protocol is generally applicable to all plants susceptible to *Agrobacterium*-mediated transformation, which, besides *Arabidopsis*, was also demonstrated for several crops, including tomato, rice, maize, wheat and barley<sup>16–20</sup>. However, with some adjustments in the transformation procedure, it should be possible to use *Agrobacterium*-independent methods as well<sup>10</sup>. Thus, this protocol is not restricted to specific plant species. The same is true for plants that require regeneration steps and varying numbers of generations to establish the chromosomal rearrangement on each allele. In the case of polyploid plants, additional junction-specific PCRs might be required. With respect to protospacer adjacent motif (PAM) and Cas nuclease preferences, other Cas enzymes, such as Cas12a, might also be applicable in this protocol<sup>21</sup>. Although only described for inversions and chromosome arm translocations, this protocol is applicable to other chromosomal rearrangements as well, such as deletions, intrachromosomal translocations or chromosome fusions and also those requiring the induction of more than two DSBs<sup>2</sup>.

### Limitations

We used the protocol described here to successfully generate and select chromosomal rearrangements in the model plant *A. thaliana*. However, this process might be more challenging in crop plants, considering the complexity of their genomes. In contrast to crops, the genome of *Arabidopsis* is relatively small, with a diploid chromosome set of only a few chromosomes. Crop genomes can be polyploid and orders of magnitude larger. Thus, multiple DSBs might cause unintended chromosomal rearrangements between subgenomes. Here, more attention needs to be paid in terms of sequence divergence between the subgenomes. In addition, further junction-specific PCRs might be required. Even though it was speculated that large-scale chromosomal rearrangements of multiple Mbp might suffer from low frequencies, just recently, an inversion of 75.5 Mbp could be generated in maize, demonstrating that those types of rearrangements are also feasible in more complex crop plants<sup>10</sup>.

As this protocol's PCR-based selection process for chromosomal rearrangements is likely transferable to other plants, limitations will primarily be encountered in the nonscreening part of the protocol. In the case of *A. thaliana*, it was shown that SaCas9 is the most efficient Cas9 in terms of mutation rates and, thus, has been used for the induction of chromosomal rearrangements so far<sup>4–6,22</sup>. In other plants, other Cas9 variants may be more suitable in this respect<sup>21</sup>, but it is essential that cleavage of the respective target sites occurs with high efficiency. The cleavage efficiency can be determined in pre-experiments by analyzing the mutation rate at the target site, which serves as proxy for the cutting efficiency. The mutation rate can be determined using the TIDE tool (<https://tide.nki.nl/>) as described in 'Experimental design' under 'Selection of target sites'. Mutation rates of ~50% or higher are desirable. Furthermore, the choice of the promoter used for Cas9 expression plays a crucial role as it strongly affects germline transmission and, thus, the frequency of chromosomal rearrangements. In *Arabidopsis*, the egg-cell-specific promoter turned out to be optimal to guarantee germline transmission for a variety of genomic changes<sup>4–6,12,14,23</sup>. Somatic promoters can induce chromosomal rearrangements in somatic cells but impede germline transmission. In plants that allow regeneration from protoplasts or embryos, in vitro assembled ribonucleoproteins might bypass the choice of promoters<sup>10</sup>. In most crop plants, transformation could be another bottleneck hindering the induction of chromosomal rearrangements<sup>24</sup>. In *Arabidopsis*, adult plants can be transformed efficiently by *Agrobacterium*-mediated T-DNA integration. However, most crops are not susceptible to efficient *Agrobacterium*-mediated transformation or require regeneration procedures<sup>24</sup>. In certain plants, such as monocots, incorporation of morphogenic regulators might be necessary to facilitate regeneration and, thus, generation of plants with chromosomal rearrangements<sup>10,25</sup>.

### Comparison with other methods

Recently, the first Mbp-sized inversion in a crop plant, namely maize, was described that spans one-third of the respective chromosome<sup>10</sup>. This was achieved by an approach similar to the one described here. In contrast to our protocol in *Arabidopsis*, immature embryos were transformed by pre-assembled ribonucleoprotein complexes via particle bombardment. In addition, instead of SaCas9, Cas9 from *Streptococcus pyogenes* was used for DSB induction. Similar to our approach, Schwartz et al. at first evaluated the mutation rates at the potential target sites prior to the experiment and selected the ones with the highest mutation frequencies. In the actual experiment, regenerated plants were analyzed by junction-specific PCRs. However, instead of using a bulk screening, individual plants were analyzed. In this way, 1,500 plants were analyzed in total. Using our bulk screening approach for *Arabidopsis*, instead of >1,500 samples, we only had to analyze <250 samples for a similar outcome. We suggest that a bulk screening should generally be applied to facilitate the screening process. It was evident that only in *Arabidopsis* did perfect re-ligations of the cut DNA ends occur at both junctions, resulting in an error-free, 'perfect' inversion. Also, more independent plant lines carried the inversion in *Arabidopsis*<sup>5</sup>. The few available studies on large-scale chromosomal rearrangements show that analyzing the cleavage efficiencies at the target sites and screening a high number of plants are crucial steps to successfully establishing chromosomal rearrangements in plants.

### Experimental design

#### Selection of target sites

Before the start of the experiment, and regardless of the type of chromosomal rearrangement, appropriate target sites need to be identified and tested for the cleavage efficiency of Cas9 at these sites. In the case of a euchromatic target region, two to three target sites per cut site are usually

sufficient to identify at least one target site with high Cas9 cutting efficiency. In the case of a heterochromatic region, a larger number of target sites has to be tested to compensate for the reduced accessibility. We recommend testing three to six target sites in a heterochromatic region. Depending on how freely the position of the DSB can be chosen in the respective target region, the tested target sites can be several hundred kilobases apart. Per target site, ten *A. thaliana* plants are transformed with the respective T-DNA construct. Mutation rates at the target site are analyzed by separately extracting the DNA of ten individual T1 lines, amplifying the target site by PCR and Sanger sequencing of the amplicons. The mutation rates are determined using the TIDE tool (<https://tide.nki.nl/>). The primers for amplification and sequencing are designed based on the recommendations on <https://tide.nki.nl/>: the projected cleavage site should be located ~200 bp from the sequencing start site (e.g., from the forward primer), and the total sequencing read should be ~700 bp, enclosing the cleavage site. Therefore, we recommend a total amplicon size between 400 and 1,000 bp. The primers should have a length between 18 bp and 24 bp, a GC content between 40% and 60% and a  $T_m$  between 50 °C and 60 °C. The primer pairs should have  $T_m$  within 5 °C of each other and not have complementary regions. Mutation rates of ~50% or higher are desirable for the induction of inversions or translocations. However, for the induction of deletions, also lower mutation rates of ~30% are sufficient. The target site with the highest mutation rate is chosen for the chromosome-engineering experiment.

### Selection of the nuclease

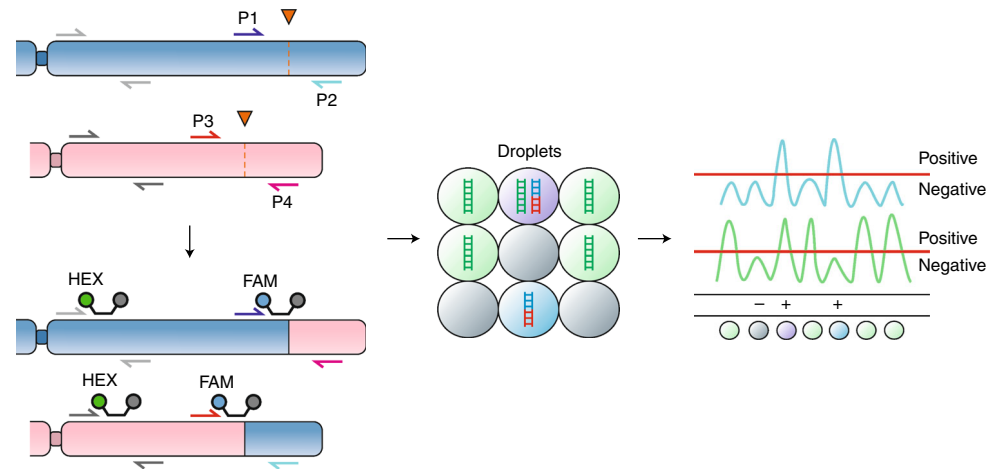
It has been shown that, by using the temperature-tolerant version of Cas12a from *Lachnospiraceae bacterium*, GT efficiencies could be enhanced in *A. thaliana* compared with the native Cas12 enzyme or SaCas9<sup>15,26</sup>. However, a similar effect has not yet been reported in the case of chromosome engineering. So far, all heritable chromosomal rearrangements in plants were induced using Cas9<sup>5,6,10</sup>. Therefore, at the moment, we recommend using SaCas9 for chromosome engineering in *A. thaliana*. This protocol describes the procedure for using the SaCas9 in combination with an egg-cell-specific promoter to promote chromosomal rearrangement transmission to the germline. When using SaCas9, we recommend using a 5'-NNGGGT-3' PAM as cleavage at those targets most likely results in the highest mutation rates<sup>22</sup>.

### Design of the gRNA

In total, two to six 20-nt-long protospacer sequences need to be selected in the target region that are accompanied by the SaCas9 PAM 5'-NNGGG-3'. The genomic sequence can be retrieved from the 1001 Arabidopsis Genomes Project genome browser (<http://signal.salk.edu/atg1001/3.0/gebrowser.php>). To avoid off-target effects, the selected protospacer sequences should be aligned with the whole *Arabidopsis* genome using the TAIR tool 'BLAST' (<https://www.arabidopsis.org/Blast/>; see Step 1). They should show a difference of at least two nucleotides to the sequences with the highest sequence identity scores. Alternatively, online CRISPR-Cas9 off-target prediction tools can be used to select suitable protospacer sequences. In our recent protocol on gRNA design, we provide further instructions<sup>27</sup>. The 20 nt protospacer sequence is designed as forward and reverse oligo with BbsI sticky ends and is integrated into the Gateway-compatible entry vector pEn-Sa-Chimera<sup>4,22,28</sup> (containing the gRNA cassette without protospacer sequence) by BbsI restriction digest and ligation. The protospacer sequence is the only part that has to be changed to adapt to any target site.

### Selection of *Arabidopsis* genetic background

It was demonstrated that by transforming the T-DNA insertion line *ku70-1* (SALK\_123114)<sup>29</sup> the frequency of chromosomal rearrangements can be increased compared with wild type<sup>4,6</sup>. The mutant lacks KU70, a key factor of classical NHEJ, which is involved in binding to the broken DNA ends, keeping the two ends linked together during the repair process and recruiting other repair proteins<sup>30</sup>. The absence of KU70 increases the probability that two previously unlinked ends are ligated by alternative NHEJ<sup>6</sup>. However, the knock-out of KU70 also leads to the formation of InDels at the newly formed junctions. For perfectly re-ligated junctions, it is preferable to transform the wild-type background. Also, the *ku70-1* T-DNA insertion line is genetically unstable if propagated over several generations<sup>31</sup>. For proof-of-concept studies, the use of the *ku70-1* mutant leads to a higher frequency of successful rearrangements. It was demonstrated that, by using the mutant line as a genetic background, translocation frequencies increased approximately fivefold compared with the wild type<sup>6</sup>. It is possible to restore the wild-type phenotype by back-crossing with the wild type.



**Fig. 3 | Principle of a probe-based duplex assay to measure translocation efficiency.** Translocation events are identified with junction-specific primers (blue, red and pink arrows labeled P1-P4). For normalization, chromosome-specific primer pairs are used (shown in gray and dark-gray arrows). Amplicons of the junction-specific PCR and the normalization PCR are categorized by FAM and HEX probes, respectively. Orange arrowheads indicate CRISPR-Cas-induced DSBs. In the middle image, green droplets indicate the normalization probe, representing the wild-type control. Blue droplets represent the translocation probe. Cyan droplets indicate that both the normalization and translocation probe were detected. Negative droplets, where no PCR reaction is detectable, are shown in gray. The right image shows a schematic overview of the droplet evaluation in a QX200 droplet reader. Each peak above the threshold (red line) is counted as positive, and each peak below the threshold is counted as negative. Blue peaks indicate that the droplets tested positive for the translocation, whereas the green peaks are positive for the normalization.

**Floral dip transformation of *A. thaliana***

Floral dip transformation<sup>32</sup> was chosen as a transformation method that allows for transformation of *A. thaliana* without the need for plant regeneration. Briefly, all seed pods and open flowers of 4–5-week-old plants are cut off. The plants are dipped in *Agrobacterium* solution for 30 s and incubated overnight in the dark. On the next day, they are transferred to the greenhouse and grown at 22 °C in 16 h light/8 h dark conditions for 4–5 weeks until seed set.

**Number of propagated plants**

For both inversions and translocations, a similar procedure is required to obtain positive plants. It is sufficient to transform 20 *A. thaliana* plants to obtain an adequate number of primary transformants, which are selected on herbicide-containing medium. Of those primary transformants, we recommend to propagate 40–80 plants. The resulting T2 lines are then subjected to bulk PCR screenings (pools of 40–80 plants per line) to identify lines carrying the chromosomal rearrangement. The individual plants carrying the rearrangement are identified by separate DNA extraction of each plant of the positively screened T2 line and subsequent PCR screening. As reciprocal translocations are more complex rearrangements than inversions, more plants have to be analyzed in the bulk screening of the T2 lines. In the case of inversions, 40 plants per line, and in the case of translocations, 80 plants per line, are pooled. The latter are screened in the form of two pools each of 40 plants.

**Design of ddPCR assay**

The overall genome-engineering efficiency can be determined by ddPCR analysis. Two different kinds of probes need to be designed according to the instructions in the Droplet Digital PCR Application Guide: junction-specific probes to detect the anticipated newly formed junctions, caused by a successful rearrangement, and normalization probes as wild-type control (Fig. 3). The detected concentration (copies per µl) of the newly formed junction is divided by the concentration of the wild-type junction to quantify the percentage of chromosomal rearrangements. The ddPCR assay is established by cloning and testing control constructs to ensure the specificity of the primers and probes for the rearrangement. The control constructs contain a sequence consisting of the anticipated junctions, flanked upstream and downstream by a 200–250 bp sequence. Also, a restriction enzyme site is added in the middle of the junction that is not present in the target sequence. The cloning is performed via Gibson Assembly<sup>33</sup>. To perform an efficient ddPCR assay, correct primer and probe

design is crucial. The GC content of the target should amount to 40–60%. The amplicon size should range between 60 and 200 bp. Primers should have a GC content between 50% and 60% and a  $T_m$  between 50 °C and 65 °C. If possible, G or C repeats larger than 3 bp should be avoided. Also, secondary structures and primer dimers should be avoided. Preferably, the probe is located in the center of the amplicon. Ensure that the probe does not overlap with the primer sequence. The GC content of the probe should range between 30% and 80% and contain more Cs than Gs. The  $T_m$  should be 3–10 °C higher than that of the primers and should consist of less than 30 nucleotides. If possible, probes with a G located at the 5' end should be avoided. At the 3' end, a Black Hole Quencher or another nonfluorescent quencher modification is added. When the ddPCR assay has been optimized for the control constructs, the experiment can be performed with the respective DNA samples under the same conditions. For further information, follow the instructions of the Droplet Digital PCR Application Guide on the design of primers and probes.

### Controls

It is not necessary to transform the plants with a control construct. However, the ddPCR assay is optimized using control constructs as described above.

## Materials

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### Reagents

#### Vector construction

- Acetic acid (Carl Roth, cat. no. 6755)
- Agarose (VWR, cat. no. 43879)
- BbsI-HF (New England Biolabs, cat. no. R3539; supplied with 10× CutSmart Buffer)
- Boric acid (Carl Roth, cat. no. 6943)
- DNA gel loading dye (any brand)
- dNTP mix, 10 mM each (Thermo Fisher Scientific, cat. no. R0191)
- DreamTaq DNA polymerase (Thermo Fisher Scientific, cat. no. EP0705; supplied with 10× DreamTaq Buffer)
- Ethidium bromide (Carl Roth, cat. no. 7870) **! CAUTION** Ethidium bromide may cause DNA mutation; wear gloves and dispose of according to laboratory safety instructions.
- Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific, cat. no. 11791020)
- Glycerol (Carl Roth, cat. no. 3783.2)
- Instant Sticky-end Ligase Master Mix (New England Biolabs, cat. no. M0370)
- MluI-HF (New England Biolabs, cat. no. R3198)
- PCR primers or oligos used in this study are listed in Table 1 (Eurofins Genomics)
- pDe-Sa-Cas9 and pDe-Sa-Cas9 EC (described in refs. <sup>4,22,28</sup>; aliquots available upon e-mail request)
- pEn-Sa-Chimera (described in refs. <sup>4,22,28</sup>; aliquots available upon e-mail request)
- peqGOLD Gel Extraction Kit (VWR International, cat. no. 13-2500)
- peqGOLD MicroSpin Cycle-Pure Kit (VWR International, cat. no. 13-6293)
- peqGOLD Plasmid Miniprep Kit I (VWR International, cat. no. 13-6943)
- Proteinase K (Thermo Fisher Scientific, cat. no. EO0491)
- Q5 High-Fidelity DNA Polymerase (New England Biolabs, cat. no. M0491; supplied with 5× Q5 Reaction Buffer)
- T4 DNA ligase (Thermo Fisher Scientific, cat. no. EL0011; supplied with 10× T4 DNA Ligase Buffer)
- Thermo Scientific GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, cat. no. SM0312)
- Tris (Carl Roth, cat. no. 4855)

#### Cultivation of *E. coli*

- Ampicillin sodium salt (Carl Roth, cat. no. K029)
- Sodium chloride (NaCl; any brand)
- Plant agar (Duchefa, cat. no. P1001)
- Spectinomycin HCl pentahydrate (Duchefa, cat. no. S0188)
- Tryptone (Duchefa, cat. no. T1332)
- Yeast extract (Duchefa, cat. no. Y1333)

**Table 1 | Oligonucleotides used**

Step 2	Forward oligo	attgNNNNNNNNNNNNNNNNNNNN	Oligo for gRNA entry vector construction
	Reverse oligo	aaacNNNNNNNNNNNNNNNNNNNN	Oligo for gRNA entry vector construction
Steps 72, 120	Forward primer	NNNNNNNNNNNNNNNNNNNN	Forward primer for TIDE analysis; forward (P1/P3) or reverse primer (P3) for T2 screening (Fig. 5)
	Reverse primer	NNNNNNNNNNNNNNNNNNNN	Reverse primer for TIDE analysis; forward (P2) or reverse primer (P2/P4) for T2 screening (Fig. 5)
Step 84	Forward primer	NNNNNNNNNNNNNNNNNNNN	Forward primer for amplification of ddPCR control construct
	Reverse primer	NNNNNNNNNNNNNNNNNNNN	Reverse primer for amplification of ddPCR control construct
	Oligo	NNNNNNNNNN*NNNNNNNNNN	ddPCR control probe; the asterisk represents a restriction enzyme site added in the middle of the probe
Steps 90, 107	Forward primer	NNNNNNNNNNNNNNNNNNNN	Forward primer for amplification of ddPCR junction-specific probe
	Reverse primer	NNNNNNNNNNNNNNNNNNNN	Reverse primer for amplification of ddPCR junction-specific probe
	Oligo	NNNNNNNNNNNNNNNNNNNN	ddPCR junction-specific probe
	Forward primer	NNNNNNNNNNNNNNNNNNNN	Forward primer for amplification of ddPCR normalization probe
	Reverse primer	NNNNNNNNNNNNNNNNNNNN	Reverse primer for amplification of ddPCR normalization probe
Step 97	Oligo	NNNNNNNNNNNNNNNNNNNN	ddPCR normalization probe
	LM73	TTTGTACAAAAAGCAGGCT	Forward primer for colony PCR after ligation of the first gRNA cassette
Steps 32, 97, 100, 104	SS144	GTCCGGACGTCTTAATTAACC	Reverse primer for colony PCR after ligation of the first gRNA cassette
Steps 10, 17, 20	SS42	TCCCAGGATTAGAATGATTAGG	Forward primer for colony PCR and sequencing
Step 20	SS102	CACCATGTTATCACATCAATCC	Reverse primer for colony PCR after Gateway reaction
Step 140	CS331	CTTGTATTCAGTAACATTTGCAGCT	Forward primer for Cas9 screening
	JS252	CTTCTCTAGCAAGGTGGAGC	Reverse primer for Cas9 screening

Lowercase letters represent overhangs. Ns represent the target-specific sequence.

### Cultivation of *A. tumefaciens*

- Beef extract (GERBU, cat. no. 1651)
- Gentamycin sulfate (Duchefa, cat. no. G0124)
- Magnesium sulfate heptahydrate (Merck, cat. no. 105886)
- Peptone (Duchefa, cat. no. P1328)
- Plant agar (Duchefa, cat. no. P1001)
- Rifampicin (Duchefa, cat. no. R0146)
- Spectinomycin HCl pentahydrate (Duchefa, cat. no. S0188)
- Sucrose (any brand)
- Yeast extract (Duchefa, cat. no. Y1333)

### Cultivation of *A. thaliana*

- Gentamycin sulfate (Duchefa, cat. no. G0124)
- Kanamycin sulfate (Carl Roth, cat. no. T832)
- L-Phosphinothricin (PPT; Duchefa, cat. no. P0159)
- Murashige and Skoog medium including vitamins/MES buffer (Duchefa, cat. no. M0255)
- Plant agar (Duchefa, cat. no. P1001)
- Sodium hypochlorite (NaClO), 12% (wt/vol) (Carl Roth, cat. no. 9062)
- Substrate (Florigard, cat. no. FG115464)
- Sucrose (any brand)
- Vermiculite, 2–3 mm (any brand)

### Transformation of *A. thaliana*

- 4'-Hydroxy-3',5'-dimethoxyacetophenon (Acetosyringone; Sigma-Aldrich, cat. no. D134406)
- 6-Benzylaminopurine (BAP; Duchefa, cat. no. B0904)



- Murashige and Skoog medium including vitamins/MES buffer (Duchefa, cat. no. M0255)
- Silwet Gold (Hermann Meyer KG, cat. no. 800147)
- Sucrose (any brand)
- Potassium hydroxide (KOH; any brand)

#### DNA extraction

- EDTA (any brand)
- Hydrogen chloride (HCl; any brand)
- Lithium chloride (LiCl; Carl Roth, cat. no. P007)
- Sodium dodecyl sulfate (Carl Roth, cat. no. CN30)
- Tris-hydrochloride (Tris-HCl; Promega, cat. no. H5121)
- ddPCR
- Automated Droplet Generation Oil for Probes (Bio-Rad Laboratories, cat. no. 1864110)
- Diluent B (New England Biolabs, cat. no. B8002S)
- Primers (Eurofins Genomics; see 'Experimental design' for further information on primer design)
- Probes (biomers; see 'Experimental design' for further information on primer design)

#### Biological materials

- *Agrobacterium tumefaciens* GV3101 (pMP90) (any brand)
- *Arabidopsis thaliana* Columbia (Col-0) and *ku70-1* T-DNA insertion line (SALK\_123114)<sup>29</sup>
- One Shot ccdB Survival 2 T1R Competent Cells (Thermo Fisher Scientific, cat. no. A10460)
- NEB 5-alpha Competent *E. coli* (High Efficiency) (Thermo Fisher Scientific, cat. no. C2987)

#### Equipment

- ddPCR 96-well plates (Bio-Rad Laboratories, cat. no. 12001925)
- ddPCR droplet reader oil (Bio-Rad Laboratories, cat. no. 1863004)
- ddPCR supermix for probes (Bio-Rad Laboratories, cat. no. 1863024)
- DG32 automated droplet generator cartridges (Bio-Rad Laboratories, cat. no. 1864109)
- PCR plate heat seal, foil, pierceable (Bio-Rad Laboratories, cat. no. 1814040)
- Pipette tips for the AutoDG System (Bio-Rad Laboratories, cat. no. 186-4120)
- Aluminum foil (any brand)
- Beaker, 1 L (any brand)
- Centrifuge bottles, 500 ml (any brand)
- Centrifuge with vortexer (neoLab, cat. no. 7-0040)
- Clean bench
- Cooling centrifuge with adapters for 500 ml centrifuge bottles (any brand)
- Cultivation trays, with and without drain holes (any brand)
- Cultivation tubes for bacteria (any brand)
- Electroporation system and electroporation cuvettes (any brand)
- Flasks, 500 ml and 1 L (any brand)
- Gel documentation system (any brand)
- Growth room or growth chamber (any brand)
- Incubator at 37 °C and 28 °C (any brand)
- Magnetic stir bar (any brand)
- Magnetic stirrer (any brand)
- Microcentrifuge (any brand)
- Nanodrop (any brand)
- Paper towels (any brand)
- Petri dishes, 92 × 16 mm without ventilation cams (Sarstedt, cat. no. 82.1472.001)
- Petri dishes, 92 × 16 mm with ventilation cams (Sarstedt, cat. no. 82.1473.001)
- Petri dishes, 150 × 20 mm with ventilation cams (Sarstedt, cat. no. 82.1184.500)
- pH meter (any brand)
- Plant pots, 5 × 5 cm (any brand)
- Precision scale (any brand)
- PX1TM PCR plate sealer (Bio-Rad Laboratories, cat. no. 1814000)
- QX200 Automated Droplet Generator (Bio-Rad Laboratories, cat. no. 1864100)
- QX200 Droplet Reader (Bio-Rad Laboratories, cat. no. 1864003)

- Shaking incubator at 37 °C and 28 °C (any brand)
- Thermal cycler (any brand)
- Ultrapure water system (any brand)

#### Software and other resources

- 1001 Arabidopsis Genomes Project genome browser (<http://signal.salk.edu/atg1001/3.0/gebrowser.php>).
- ApE—A Plasmid Editor v2.0.55 (M. Wayne Davis, Salt Lake City, Utah, USA)
- BioRad. Droplet Digital™ PCR Applications Guide ([https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin\\_6407.pdf](https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf))
- NEBuilder HiFi DNA Assembly (<https://www.neb-online.de/en/cloning-synthetic-biology/dna-assembly/nebuilder-dna-assembly/>)
- QuantaSoft Analysis Pro Software 1.0 (Bio-Rad Laboratories GmbH, München)
- TAIR—The Arabidopsis Information Resource (<http://www.arabidopsis.org/>)
- TIDE (<https://tide.nki.nl/>; <sup>34</sup>)

#### Reagent setup

All media were prepared using ddH<sub>2</sub>O.

#### 20× sodium borate (SB) buffer

8 g/L NaOH, 47 g/L boric acid, pH 8.0. SB buffer can be stored at room temperature (RT, 20–25 °C) for several months.

#### 50× Tris acetate EDTA (TAE) buffer

242 g/L Tris, 57.1 ml/L glacial acetic acid, 100 ml/L 0.5 M EDTA, pH 8.0. TAE buffer can be stored at RT for several months.

#### Growth medium (GM)

Mix 4.9 g/L Murashige and Skoog medium with 10 g/L sucrose; 8 g/L plant agar for solid media; adjust to pH 5.7 with KOH. Autoclave. GM medium can be stored at RT or 4 °C for several months.

#### Infiltration medium

Mix 2.5 g/L Murashige and Skoog medium with 50 g/L sucrose. Adjust to pH 5.7 using KOH. Add 4.25 µl/L 0.1% (wt/vol) BAP. Immediately before transformation, add 1 ml/L 10% (vol/vol) Acetosyringone and 0.5 ml/L Silwet Gold. Infiltration medium can be prepared the day before transformation and stored at 4 °C overnight or prepared fresh on the day of transformation.

#### Lysogeny broth (LB) medium

Mix 10 g/L tryptone with 5 g/L yeast extract and 5 g/L NaCl; 17.5 g/L plant agar for solid media. Autoclave. LB medium can be stored at RT or 4 °C for several months.

#### DNA extraction buffer

200 mM Tris-HCl (pH 9.0), 400 mM LiCl, 25 mM EDTA, 1% (wt/vol) sodium dodecyl sulfate, pH 9.0. Shorty DNA extraction buffer can be stored at RT for several months.

#### Tris-EDTA (TE) buffer

10 mM Tris-HCl (pH 9.0), 1.0 mM EDTA; adjust to pH 8.0 with HCl. TE buffer can be stored at RT for several months.

#### Yeast extract beef (YEB) medium

Mix 5 g/L beef extract with 5 g/L peptone, 1 g/L yeast extract, 5 g/L sucrose and 493 mg/L MgSO<sub>4</sub>; 12 g/L plant agar for solid media. Autoclave. YEB medium can be stored at RT for several months.

## Procedure

**Stage 1: determination of Cas9 mutation rates as proxy for cutting efficiency at the target sites****Design of gRNAs ● Timing 1 d**

- 1 Select two to six 20-nt-long protospacer sequences in the target region that are accompanied by the SaCas9 PAM 5'-NNGGG-3'. The genomic sequence can be retrieved from the 1001 Arabidopsis Genomes Project genome browser (<http://signal.salk.edu/atg1001/3.0/gebrowser.php>). To avoid off-target effects, align the selected protospacer sequences with the whole *Arabidopsis* genome using the TAIR tool 'BLAST' (<https://www.arabidopsis.org/Blast/>). In the BLAST menu, select the BLAST program 'BLASTN'. In the datasets menu, select 'TAIR 10 Whole Genome (BAC clones) (DNA)'. Copy-paste your selected protospacer sequence into the input field. Select 'Run BLAST'. Choose protospacer sequences that show a of at least two nucleotides to the sequences with the highest sequence identity scores. Alternatively, use online CRISPR-Cas9 off-target prediction tools. Follow our recent protocol on gRNA design for further instructions<sup>27</sup>.
- 2 Order forward and reverse oligos with BbsI sticky-end overhangs for each gRNA for subsequent ligation with the BbsI-digested backbone vector pEn-Sa-Chimera (Table 1). Order the oligos unmodified at a synthesis scale of 0.01 μmol, lyophilized and desalted.  
**▲ CRITICAL STEP** For subsequent ligation of the protospacer sequence with the BbsI-digested backbone pEn-Sa-Chimera, the forward and reverse oligos need to be annealed (see Steps 6–7) to obtain a double-stranded DNA sequence with complementary BbsI sticky-end overhangs (ATTG at the 5' end of the forward oligo and AAAC at the 5' end of the reverse oligo).  
**■ PAUSE POINT** Resuspended oligos can be stored at –20 °C for years.

**Construction of the Gateway entry vector pEn-Sa-Chimera ● Timing 3 d**

- 3 Digest pEn-Sa-Chimera with BbsI in CutSmart Buffer at 37 °C for 1.5 h as follows:

Component	Amount (μl)	Final concentration
10× CutSmart Buffer	5	1×
pEn-Sa-Chimera (100 ng/μl)	10	20 ng/μl
BbsI (20 U/μl)	1	2.5 U/μl
ddH <sub>2</sub> O	34	
Total	50	

- 4 Purify the digested vector using the Cycle-Pure kit according to the manufacturer's instructions.
- 5 Resuspend oligos (from Step 2) with ddH<sub>2</sub>O to obtain a final concentration of 50 pmol/μl.
- 6 Mix 2 μl of forward and reverse oligo with ddH<sub>2</sub>O to obtain a total volume of 50 μl.

Component	Amount (μl)	Final concentration
Forward oligo (50 pmol/μl)	2	2 pmol/μl
Reverse oligo (50 pmol/μl)	2	2 pmol/μl
ddH <sub>2</sub> O	46	
Total	50	

- 7 Heat a PCR cycler to 95 °C. Denature the oligos at 95 °C for 5 min. Immediately afterward take the samples out of the cycler, and let them slowly cool down to RT for 20 min.

Step	Condition
1	95 °C, hold
2	95 °C for 5 min
3	Cool at RT for 20 min

- ▲ CRITICAL STEP** The annealed oligos form a double-stranded protospacer harboring BbsI overhangs complementary to the sticky ends of the digested pEn-Sa-Chimera.  
**■ PAUSE POINT** Annealed oligos can be stored at –20 °C for several months.

- To ligate the BbsI-digested entry vector pEn-Sa-Chimera with the annealed oligos, carry out a ligation reaction as follows using the Instant Sticky-end Ligase Master Mix. No incubation time is required.

Component	Amount (μl)	Final concentration
Digested pEn-Sa-Chimera (5 ng/μl) from Step 3	2	1 ng/μl
Annealed oligos from Step 7	3	
2× Instant Sticky-end Ligase Master Mix	5	1x
Total	10	

**▲ CRITICAL STEP** According to the manufacturer’s instructions, no incubation time is required for ligation of sticky ends between 2 nt and 4 nt.

**■ PAUSE POINT** Ligation reaction can be stored at –20 °C for weeks.

- Transform 100 μl of NEB 5-alpha *E. coli* cells with 5 μl of the ligation reaction using a standard heat shock-transformation protocol (in brief: thaw 100 μl NEB 5-alpha on ice and add to 5 μl of the ligation reaction, incubate for 20 min on ice, heat shock for 45 s at 42 °C, immediately transfer back to ice and incubate for 2 min, add 500 μl LB medium to the cells, incubate for 1 h at 37 °C on a shaker at 500 rpm, spread 100 μl of transformation reaction on a LB plate containing 100 mg/L ampicillin and incubate overnight at 37 °C).

**■ PAUSE POINT** After overnight incubation, plates can be stored at 4 °C for a few days to weeks.

**? TROUBLESHOOTING**

- To confirm successful integration of the protospacer into the backbone, prepare a colony PCR mastermix using the primer SS42 (Table 1) and the reverse oligo from Step 2. SS42 binds to the U6-26 promoter in the backbone pEn-Sa-Chimera. The PCR can only yield a product with the primer combination SS42/reverse oligo if the protospacer has been integrated successfully. Prepare the master mix in bulk and aliquot 25 μl into each PCR tube. Prepare 10–20 PCR tubes (equal to the number of colonies to be tested).

Component	Amount (μl)	Final concentration
10× DreamTaq Buffer	2.5	1x
dNTP mix (10 μM)	0.5	2.5 μM
Forward primer SS42 (50 pmol/μl)	0.25	0.5 pmol/μl
Reverse oligo from Step 2 (50 pmol/μl)	0.25	0.5 pmol/μl
DreamTaq polymerase (5 U/μl)	0.13	0.026 U/μl
ddH <sub>2</sub> O	21.37	
Total	25	

- As template, pick 10–20 colonies from the plate from Step 9 with pipette tips, and transfer the whole colonies to a new LB plate containing 100 mg/L ampicillin for inoculation of new overnight cultures in Step 14 (with the colonies identified as positive in the colony PCR in Step 13) and then add each pipette tip directly to a PCR tube containing the master mix from Step 10. Stir the pipette tip in the PCR tube for 3 s, then discard the pipette tip. Incubate the new LB plate for at least 1.5 h at 37 °C to allow subsequent inoculation of overnight cultures in Step 14 with the colonies identified as positive in the colony PCR (in Step 13).
- Perform the colony PCR program as follows:

Cycle	Denature	Anneal	Extend	Hold
1	95 °C, 3 min			
2–30	95 °C, 20 s	56 °C, 20 s	72° C, 30 s	
31			72 °C, 1 min	
32				12 °C, hold

**■ PAUSE POINT** PCR reactions can be stored at 4 °C for a few days.

- 13 Add gel loading dye to the PCR reaction and load 15  $\mu\text{l}$  on a 0.9% (wt/vol) SB agarose gel containing 1% (wt/vol) ethidium bromide in 1 $\times$  SB buffer. Load 2.5  $\mu\text{l}$  of a DNA ladder. Run in SB buffer for 20–30 min at 200 V. The expected amplicon has a size of 284 bp (SS42 binds in the backbone, therefore the distance between SS42 and the reverse oligo is always the same, independent of the target site).
- 14 Pick one to two clones per construct from the new LB plate set up in Step 11 that were determined to be positive by gel electrophoresis in Step 13, and inoculate each clone separately into 5 ml of LB medium containing 100 mg/L ampicillin in cultivation tubes. Incubate overnight at 37 °C in a shaker at 200 rpm.
- 15 (Optional) To obtain glycerol stocks, mix 700  $\mu\text{l}$  of the culture with 300  $\mu\text{l}$  99.9% (vol/vol) glycerol under sterile conditions to be able to store the cells at –80 °C for years.
- 16 Extract plasmid DNA from 4 ml of the overnight culture using the Plasmid Miniprep Kit I according to the manufacturer's instructions.  
■ **PAUSE POINT** Plasmids can be stored at –20 °C for years.
- 17 Confirm successful ligation by Sanger sequencing using the primer SS42 (Table 1).

### Construction of the Gateway destination vector pDe-Sa-Cas9 ● Timing 3 d

- 18 After confirmation of successful ligation by sequencing, choose one positive clone and add to the LR Gateway reaction mastermix with the destination vector pDe-Sa-Cas9, as follows:

Component	Amount ( $\mu\text{l}$ )	Final concentration
pEn-Sa-Chimera from Step 14 (100 ng/ $\mu\text{l}$ )	2	20 ng/ $\mu\text{l}$
pDe-Sa-Cas9 (50 ng/ $\mu\text{l}$ )	3	15 ng/ $\mu\text{l}$
TE buffer (pH 8.0)	4	
LR clonase II Enzyme Mix	1	
Total	10	

Incubate for 2–3 h at 25 °C. Stop reaction by adding 1  $\mu\text{l}$  of Proteinase K, and incubate at 37 °C for 10 min.

■ **PAUSE POINT** Reaction can be stored at –20 °C for a few weeks.

- 19 Transform 100  $\mu\text{l}$  of *E. coli* NEB 5-alpha with the reaction. Briefly, add the whole reaction to 100  $\mu\text{l}$  of competent cells, incubate for 20 min on ice, heat shock at 42 °C for 90 s, transfer back on ice for 2 min, add 500  $\mu\text{l}$  LB medium and incubate for 1 h at 37 °C on a shaker at 650 rpm. Plate 100  $\mu\text{l}$  of the transformation reaction on a LB plate containing 100 mg/L spectinomycin. Incubate at 37 °C overnight.  
? **TROUBLESHOOTING**
- 20 (Optional) Perform a colony PCR according to Step 10, using the primers SS42 and SS102 (Table 1). SS102 binds to the kanamycin resistance cassette in the backbone vector. Use the following PCR program:

Cycle	Denature	Anneal	Extend	Hold
1	95 °C, 3 min			
2–30	95 °C, 20 s	60 °C, 20 s	72 °C, 1 min	
31			72 °C, 1 min	
32				12 °C, hold

- 21 Repeat Step 13. The expected amplicon has a size of 856 bp (SS102 binds in the backbone; therefore the distance between SS42 and SS102 is always the same, independent of the target site).
- 22 Choose one or two positive clones, as confirmed by colony PCR in Step 21 or, if Steps 20 and 21 were not carried out, pick one or two random colonies and inoculate each into 5 ml of LB medium containing 100 mg/L spectinomycin. Incubate at 37 °C on a shaker overnight.
- 23 Repeat Steps 15–17.

**Transformation of *Agrobacterium* with destination vector ● Timing 3 d**

- 24 Thaw electrocompetent *Agrobacterium tumefaciens* GV3101 on ice.
- 25 Mix 1 µl of the destination vector from Step 21 with 50 µl of *A. tumefaciens* GV3101 in an electroporation cuvette.
- 26 Pulse at 2,500 V, 25 µF und 200 Ω for 5 ms using an electroporation system.
- 27 Transfer cuvette back on ice.
- 28 Add 800 µl of YEB medium to each sample.
- 29 Mix by pipetting, and transfer the whole sample to a new 1.5 ml microcentrifuge tube.
- 30 Incubate for 2 h at 28 °C on a shaker at 500 rpm.
- 31 Plate 50–100 µl on YEB plates containing 100 mg/L rifampicin, 20 mg/L gentamycin and 100 mg/L spectinomycin, and incubate at 28 °C for 2 d.
  - **PAUSE POINT** Plates can be stored at 4 °C for up to 1 week.
  - ▲ **CRITICAL STEP** Plated volume depends on the transformation efficiency of the competent cells. Spectinomycin resistance is derived from the destination vector. Gentamycin and rifampicin resistance are derived from the helper plasmid and chromosomal background, respectively.
  - ? **TROUBLESHOOTING**
- 32 After 2 d, the integration of the plasmid can optionally be confirmed by a colony PCR. As forward primer, use the forward oligo from Step 2, and as a reverse primer, the primer SS144 (Table 1). Prepare the master mix in bulk and aliquot 25 µl into each PCR tube. Prepare five PCR tubes (equal to the number of colonies to be tested).

Component	Amount (µl)	Final concentration
10× DreamTaq Buffer	2.5	1×
dNTP mix (10 µM)	0.5	2.5 µM
Forward oligo from Step 2 (50 pmol/µl)	0.25	0.5 pmol/µl
SS144 (50 pmol/µl)	0.25	0.5 pmol/µl
DreamTaq polymerase (5 U/µl)	0.13	0.026 U/µl
ddH <sub>2</sub> O	21.37	
Total	25	

- 33 As template, pick five colonies from the plate from Step 31 with pipette tips, transfer the whole colonies to a new YEB plate containing 100 mg/L rifampicin, 20 mg/L gentamycin and 100 mg/L spectinomycin for subsequent inoculation of overnight cultures in Step 36 (with the colonies identified as positive in Step 35) and then add each pipette tip directly to a PCR tube containing the master mix from Step 32. Stir the pipette tip in the PCR tube for 3 s, then discard the pipette tip. Incubate the new YEB plate for 1 d at 28 °C to allow subsequent inoculation of overnight cultures in Step 36 with the colonies identified as positive in the colony PCR (Step 35).
- 34 Perform the colony PCR program as follows:

Cycle	Denature	Anneal	Extend	Hold
1	95 °C, 3 min			
2–30	95 °C, 20 s	56 °C, 20 s	72° C, 30 s	
31			72 °C, 1 min	
32				12 °C, hold

- **PAUSE POINT** PCR reactions can be stored at 4 °C for a few days.
- 35 Add gel loading dye to the PCR reaction, and load 15 µl on a 0.9% (wt/vol) SB agarose gel containing 1% (wt/vol) ethidium bromide in 1× SB buffer. Load 2.5 µl of a DNA ladder. Run in SB buffer for 20–30 min at 200 V. The expected amplicon has a size of 211 bp (SS144 binds in the desination vector backbone; therefore the distance between the forward oligo and SS144 is always the same, independent of the target site).
- 36 Pick a single positive colony from the plate from Step 33 (or a random single colony from Step 31 if no colony PCR was performed) to inoculate 10 ml of YEB medium containing 100 mg/L rifampicin, 20 mg/L gentamycin and 100 mg/L spectinomycin. Incubate overnight at 28 °C in a shaker at 200 rpm.

- 37 Prepare glycerol stocks by mixing 1 ml of the overnight culture with 500  $\mu$ l of 99.9% (vol/vol) glycerol under sterile conditions.  
■ **PAUSE POINT** Glycerol stocks can be stored at  $-80$  °C for years.

#### Cultivation of *A. thaliana* ● Timing 4–5 weeks

- 38 Four weeks prior to the desired transformation date, transfer *A. thaliana* Col-0 seeds (enough seeds to grow ten plants per construct) to a microcentrifuge tube and add ddH<sub>2</sub>O until seeds are covered. For stratification, place at 4 °C overnight.  
39 Plant on soil (substrate and vermiculite in 1:1 ratio) and grow under 16 h light/8 h dark conditions at 22 °C in the greenhouse.  
40 Grow for 4–5 weeks until plants enter the flowering stage (for further instructions, see ref. <sup>32</sup>).

#### Floral dip transformation of *Arabidopsis thaliana* ● Timing 4 d

- 41 *Main culture (1 d before transformation)*. Inoculate 200 ml of YEB medium containing 100 mg/L rifampicin, 20 mg/L gentamycin and 100 mg/L spectinomycin with one of the glycerol stocks from Step 37 in a 500 ml flask, and cover the opening with aluminum foil. Incubate overnight at 28 °C in a shaking incubator at 200 rpm.  
42 *Preparation of infiltration medium (day of transformation)*. Prepare 400 ml of infiltration medium.  
43 Immediately before beginning the floral dip transformation, add 100 mg/L Acetosyringone and 100 mg/L Silwet Gold to infiltration medium.  
44 *Preparation of floral dip transformation (day of transformation)*. Cut off all open flowers and seed pods of the plants.  
▲ **CRITICAL STEP** The main target for transformation is the egg cell. Cutting off open flowers and pods prevents self-pollination and propagation of untransformed embryos. This greatly increases transformation efficiency (i.e., ratio of untransformed to transformed seeds).  
45 Prepare two cultivation trays, one with drain holes and one without. Cover the bottom of the tray without holes in paper towels, and spray with water.  
46 *Centrifugation (day of transformation)*. Transfer entire volume of main culture from Step 41 to 500 ml centrifuge bottles, and spin down at 20 °C for 15 min at 5,500g.  
47 Discard supernatant.  
48 Resuspend pellets in 50 ml of infiltration medium from Step 42.  
49 Add resuspended pellet to the remaining 350 ml of the infiltration medium from Step 42.  
50 Transfer *Agrobacterium* solution to a 1 L beaker, add a magnetic stir bar and mix on magnetic stirrer at RT for 1 min at 200 rpm.  
51 *Floral dip (day of transformation)*. Dip plants from Step 44 for 10–30 s into *Agrobacterium* solution from Step 50 by submerging all above-ground tissue (stirrer can be switched off).  
52 Place the plants in horizontal position on the tray without holes from Step 45.  
53 Cover with the second tray with drain holes.  
▲ **CRITICAL STEP** Covering of the plants increases transformation efficiency as *A. tumefaciens* shows higher motility and infectivity in darkness<sup>35</sup>.

#### After transformation (day after transformation)

- 54 On the day after transformation, gently spray plants with a spray bottle filled with tap water (on 'mist' setting).  
55 Set plants upright again.  
56 Place plants in a clean tray.

#### Propagation ● Timing 4–5 weeks

- 57 Transfer the plants to the greenhouse. Plants are grown under 16 h light/8 h dark conditions for 4–5 weeks at 22 °C until seed set in 5 × 5 cm pots filled with soil (substrate and vermiculite in ratio 1:1). Harvest ripe seeds after 4–5 weeks.  
■ **PAUSE POINT** Seeds can be stored in dry and dark conditions for years.  
▲ **CRITICAL STEP** Harvest when ~50% of the plants are ripe. This helps prevent too many seeds derived from new untransformed buds contributing to the batch and greatly increases overall transformation efficiency.

**Primary transformant selection ● Timing 16 d**

- 58 Sterilize T1 seeds by liquid sterilization using sodium hypochlorite. First, fill a 2 ml microcentrifuge tube with seeds to ~1/8 full and add 1 ml 4% (vol/vol) sodium hypochlorite. Mix for 8–15 min by shaking. Centrifuge for 30 s at RT at 1,000g. Take off supernatant with a pipette, and discard supernatant. Wash seeds with 1 ml sterile ddH<sub>2</sub>O. Mix for 30 s, and spin down for 30 s at RT at 1,000g. Repeat wash step three to four times. Finally, add 1 ml sterile ddH<sub>2</sub>O and place seeds at 4 °C overnight for stratification.
- 59 Mix seeds with 25 ml of sterile 0.1% (wt/vol) agarose solution (dissolved in ddH<sub>2</sub>O) in a 50 ml Falcon tube, and mix by inverting.
- 60 Pour 5 ml of seed mix onto a 150 × 20 mm GM plate containing 30 mg/L kanamycin and 500 mg/L cefotaxime, and distribute seed mixture equally on the plate by swiveling the plate. Prepare five plates this way.
- 61 Grow for 2 weeks in a growth room or growth chamber under 16 h light/8 h dark conditions at 22 °C.

▲ **CRITICAL STEP** Kanamycin resistance is derived from the destination vector. Cefotaxime prevents *Agrobacterium* contamination. Transformed plants will grow wild-type-like, whereas nontransformed plants will stop growing at the two-leaf stage.

? **TROUBLESHOOTING**

**DNA extraction ● Timing 3 h to 1 d**

- 62 Choose ten primary transformants from the plates; cut off one leaf of each plant, and place in separate 1.5 ml microcentrifuge tubes.
- 63 Afterward, crush each leaf with a pestle and mortar and add 500 µl of DNA extraction buffer to the tubes.
- 64 Centrifuge for 5 min at 10,000g at RT.
- 65 Prepare new 1.5 ml microcentrifuge tubes, and add 400 µl of isopropanol.
- 66 Transfer 400 µl of the supernatant from Step 64 to the new tubes. Mix by inverting.
- 67 Centrifuge for 10 min at 14,000g at RT.
- 68 Discard supernatant, and place tubes upside down on a paper towel.
- 69 Dry pellets overnight at RT or for 1.5 h at 37 °C.
- 70 Resuspend in 50 µl TE buffer (pH 8.0) by vortexing and placing on a shaker for at least 10 min.

■ **PAUSE POINT** Samples can be stored at –20 °C or –80 °C for years.

**Preparation of TIDE analysis for determination of cutting efficiency ● Timing 1 d**

- 71 Select primers upstream and downstream of the cut site (total amplicon size should be ~400–1,000 bp (for further instructions, see ‘Experimental design’ and <http://shinyapps.datacurators.nl/tide/>).
- 72 Amplify the DNA samples from Step 70, as well as a wild-type Col-0 sample, in a Q5 polymerase PCR as follows:

Component	Amount (µl)	Final concentration
5× Q5 Reaction Buffer	10	1×
dNTPs (10 µM)	1	0.2 µM
Forward primer (50 pmol/µl) (Table 1)	0.5	0.01 pmol/µl
Reverse primer (50 pmol/µl) (Table 1)	0.5	0.01 pmol/µl
Q5 High-Fidelity DNA Polymerase (2 U/µl)	0.5	0.02 U/µl
Template (DNA samples from Step 70 and Col-0 sample)	2	
ddH <sub>2</sub> O	35.5	
Total	50	

▲ **CRITICAL STEP** High-fidelity DNA polymerases, such as Q5 High-Fidelity DNA Polymerase, minimize PCR error and will therefore result in a more accurate determination of the mutation rate.

- 73 Use the PCR program below. Choose annealing temperature corresponding to the *T<sub>m</sub>* of the used primers. Calculate extension time based on the polymerase’s ability to extend 2–3 kb/min (for further information, see product information).



Cycle	Denature	Anneal	Extend	Hold
1	98 °C, 30 s			
2-30	98 °C, 10 s	x °C, 30 s	72 °C, x s	
31			72 °C, 2 min	
32				12 °C, hold

74 Add gel loading dye to the PCR reaction, and load 15  $\mu$ l on a 0.9% (wt/vol) SB agarose gel containing 1% (wt/vol) ethidium bromide in 1 $\times$  SB buffer. Load 2.5  $\mu$ l of a DNA ladder, e.g., GeneRuler 1 kb DNA ladder. Run in SB buffer for 20–30 min at 200 V.

■ **PAUSE POINT** PCR reaction can be stored at 4 °C or –20 °C for weeks.

75 Purify the remaining PCR product using the Cycle-Pure kit according to the manufacturer's instructions.

■ **PAUSE POINT** Samples can be stored at –20 °C for months.

76 Send to Sanger sequencing with one or both primers used for amplification.

### TIDE analysis ● Timing 1 d

77 Go to <https://tide.nki.nl/> and select 'Start TIDE'.

78 Upload the protospacer sequence, the ten sequencing samples from Step 76 and the sequenced wild-type control from Step 76. Adjust parameters by clicking 'Advanced settings' according to the instructions on the website. Click 'Update View'.

79 Download the results. For each sample, a cutting efficiency is determined based on the frequency of small InDels at the target site generated by CRISPR–Cas.

80 Calculate the mean cutting efficiency at the target site from the ten individual cutting efficiencies.

81 Choose the protospacer sequence with the highest cutting efficiency for each target site.

### ? TROUBLESHOOTING

### Stage 1 (optional): determination of rearrangement induction efficiency via ddPCRDesign of the assay ● Timing 1 d

82 For a probe-based duplex assay, design two different kinds of probes according to the instructions in 'Experimental design' and the Droplet Digital PCR Application Guide: junction-specific probes to detect the anticipated newly formed junctions and normalization probes as wild-type control (Fig. 3). The detected concentration (copies per  $\mu$ l) of the newly formed junctions is divided by the concentration of the wild-type junctions to quantify the percentage of chromosomal rearrangements.

### Cloning of control constructs ● Timing 4 d

83 Clone control constructs that contain the anticipated junctions via Gibson Assembly (see 'Experimental design' for further details). Follow the instructions of the NEBuilder HiFi DNA Assembly Guide to design Gibson Assembly primers. The *Sa*Cas9 cut site needs to be flanked on both sides by a 200–250 bp sequence, and the sequence should span the primers and probes designed for the assay. To rule out contamination of the samples, the construct needs to contain a restriction enzyme site in the middle of the junction that is not present in the PCR target sequence.

### Gradient PCRs ● Timing 4 d

84 To perform a simplex thermal gradient PCR with the control constructs from Step 83 as template to find the optimal annealing temperatures of all junction-specific and wild-type probes with their respective primer combinations (Table 1) for the ddPCR analysis, prepare the mastermix for the simplex thermal gradient PCR as follows, using filter tips:

Component	Amount ( $\mu$ l)	Final concentration
ddH <sub>2</sub> O	7.05	
2 $\times$ ddPCR Probes Supermix	11	1 $\times$
Forward primer (100 $\mu$ M)	0.2	0.91 $\mu$ M
Reverse primer (100 $\mu$ M)	0.2	0.91 $\mu$ M
Probe (10 $\mu$ M)	0.55	0.25 $\mu$ M
Template (4 ng/ $\mu$ l)	3	0.55 ng/ $\mu$ l

- 85 Vortex the mastermix thoroughly. Aliquot 22  $\mu\text{l}$  into the wells of a 96-well plate. Centrifuge the plate to remove any bubbles.
- 86 Transfer the plate from Step 85 together with a new empty plate to the droplet generator. Generate a loading scheme (indicating the filled wells of the plate from Step 85) for the droplet generator following the manual's instructions. Every well indicated in the loading scheme needs to be filled with liquid (the PCR mastermix from Step 85). The droplets are generated automatically in the QX200 Droplet Generator and transferred to the new empty PCR plate. Immediately after completion, start the PCR using the conditions described in the following step.
- ▲ CRITICAL STEP** There should always be enough tips, oil and cartridges in the droplet generator. Run the PCR immediately after droplet generation, as the droplets are sensitive to heat. Do not vortex the plate.
- 87 For the thermal gradient PCR, test a temperature range of 5  $^{\circ}\text{C}$  above and below the annealing temperature of the primers by using a 2  $^{\circ}\text{C}$  ramp rate to ensure that the correct temperature of each cycle is reached in each droplet. Perform the PCR with the following conditions:

Cycle	Denature	Anneal	Extend/stabilize droplets	Hold
1	95 $^{\circ}\text{C}$ , 10 min; 2 $^{\circ}\text{C}/\text{s}$			
2-40	94 $^{\circ}\text{C}$ , 30 s; 2 $^{\circ}\text{C}/\text{s}$	$T_A - 5^{\circ}\text{C}$ to $T_A + 5^{\circ}\text{C}$ , 1 min; 2 $^{\circ}\text{C}/\text{s}$	72 $^{\circ}\text{C}$ , 2 min; 2 $^{\circ}\text{C}/\text{s}$	
41			98 $^{\circ}\text{C}$ , 10 min; 2 $^{\circ}\text{C}/\text{s}$	
42				12 $^{\circ}\text{C}$ , hold; 2 $^{\circ}\text{C}/\text{s}$

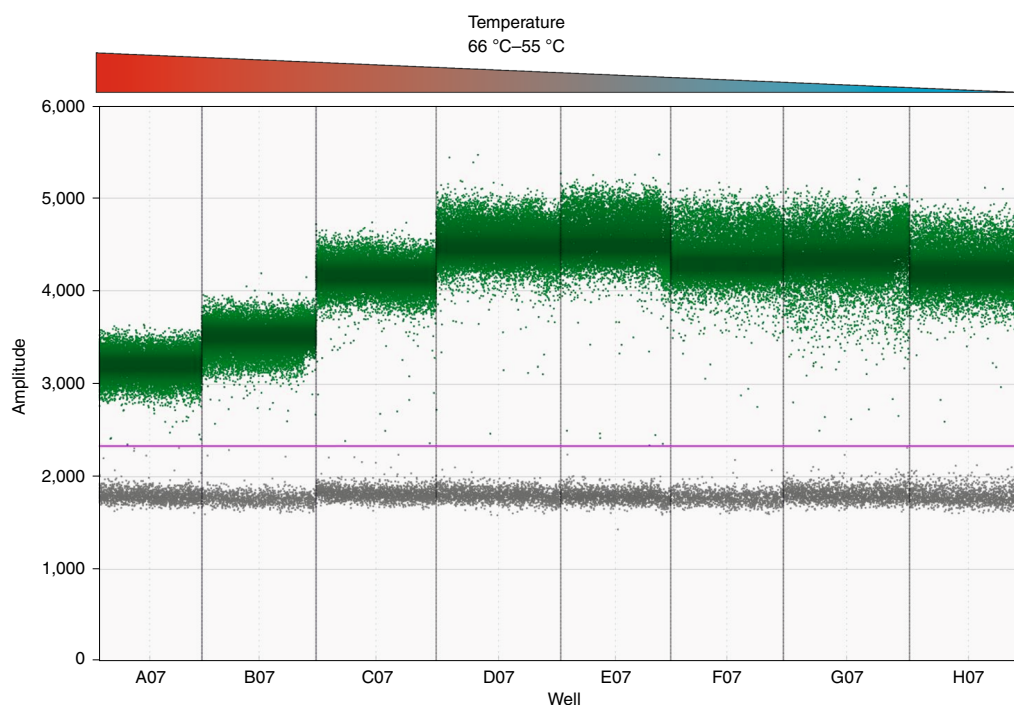
- 88 After the PCR reaction is finished, transfer the plate to the QX200 Droplet reader.
- 89 To set up and analyze the experiment using the QunataSoft Software, follow the guidelines as described in the Digital Droplet PCR Application guide. Determine the optimal annealing temperature of each probe based on which annealing temperature leads to the largest fluorescence amplitude separation of negative and positive and avoids nonspecific amplification. Negative droplets will be shown as gray, positive droplets as green (Fig. 4). For further explanations, follow the instructions of the Droplet Digital PCR Application Guide.
- 90 Afterward, perform a duplex thermal gradient PCR to determine the optimal annealing temperature for the subsequent dual-color ddPCR experiment in which two probes (junction-specific and normalization probe) will be used in the same mastermix. For a duplex thermal gradient PCR, prepare the mastermix as follows using primers and probes as detailed in Table 1.

Component	Amount ( $\mu\text{l}$ )	Final concentration
ddH <sub>2</sub> O	6.1	
2 $\times$ ddPCR Probes Supermix	11	1 $\times$
Forward primer (junction; 100 $\mu\text{M}$ )	0.2	0.91 $\mu\text{M}$
Reverse primer (junction; 100 $\mu\text{M}$ )	0.2	0.91 $\mu\text{M}$
Probe (junction; 10 $\mu\text{M}$ )	0.55	0.25 $\mu\text{M}$
Forward primer (normalization; 100 $\mu\text{M}$ )	0.2	0.91 $\mu\text{M}$
Reverse primer (normalization; 100 $\mu\text{M}$ )	0.2	0.91 $\mu\text{M}$
Probe (normalization; 10 $\mu\text{M}$ )	0.55	0.25 $\mu\text{M}$
Template (4 ng/ $\mu\text{l}$ )	3	0.55 ng/ $\mu\text{l}$

- 91 Repeat Steps 85–89.

**Cloning of selected protospacers into an ubiquitin promoter plasmid ● Timing 4–5 d**

- 92 For the induction of deletions, inversions or translocations, clone two gRNAs into a ubiquitin promoter-driven Cas construct. To introduce the first gRNA into the destination vector



**Fig. 4 | Analysis of a simplex thermal gradient PCR using the QuantaSoft Analysis Pro Software.** Thermal gradient PCR with normalization probes to detect the optimal temperature for the assay. A temperature range between 55 °C and 66 °C was tested. The pink line indicates the threshold that is set at a point where the droplets are clearly divided into two separate clusters. Every droplet above the threshold is counted as positive, every droplet below the threshold is counted as negative. The temperature at which the largest fluorescence amplitude separation of negative and positive droplets is visible is chosen for the assay (in this example between 58 °C and 60 °C). Orange arrowheads indicate CRISPR-Cas-induced DSBs. Raw data are provided in Supplementary Data 1.

pDe-Sa-Cas9, digest the destination vector and the entry vector carrying the chosen protospacer from Step 16 in two separate digests with MluI as follows. Incubate for at least 2 h or overnight at 37 °C.

Component	Amount (μl)	Final concentration
10× CutSmart Buffer	5	1×
pEn-Sa-Chimera/pDe-Sa-Cas9 (100 ng/μl)	10	20 ng/μl
MluI (10 U/μl)	1	0.2 U/μl
ddH <sub>2</sub> O	34	
Total	50	

■ **PAUSE POINT** Digested vectors can be stored at –20 °C for weeks.

93 Purify the digested pDe-Sa-Cas9 from Step 92 using the Cycle-Pure kit according to the manufacturer's instructions.

■ **PAUSE POINT** Purified digest can be stored at –20 °C for years.

94 Purify the digested pEn-Sa-Chimera from Step 92 as follows. Weigh an empty microcentrifuge tube. Add gel loading dye to the digested pEn-Sa-Chimera, and load on a 0.9% (wt/vol) TAE agarose gel containing 1% (wt/vol) ethidium bromide in 1× TAE buffer. Run at 120 V in TAE buffer for 45–60 min. Two bands should be visible on the gel. Cut out the 502 bp band using a scalpel, and add to weighed microcentrifuge tube. Use the Gel Extraction Kit to purify PCR product according to the manufacturer's instructions.

■ **PAUSE POINT** Gel piece can be stored at –20 °C for a few days.

- 95 Ligate the purified backbone pDe-Sa-Cas9 from Step 93 with the purified gRNA cassette from Step 94 in a 1:3 backbone to insert ratio using the T4 DNA ligase:

Component	Amount (μl)	Final concentration
Digested pEn-Sa-Chimera from Step 88	x	
Digested pDe-Sa-Cas9 from Step 87	x	
10x T4 DNA Ligase Buffer	2	1×
T4 DNA Ligase (5 U/μl)	2	0.5 U/μl
ddH <sub>2</sub> O	x	
Total	20	

Incubate for at least 3 h at 25 °C.

- 96 Transform *E. coli* ccdB Survival cells with the ligated vector following the transformation protocol from Step 9. Plate 100 μl on LB plates containing 100 mg/L spectinomycin. Incubate at 37 °C overnight.

▲ **CRITICAL STEP** The ligated vector still contains the ccdB cassette. Therefore, ccdB-resistant *E. coli* cells, such as ccdB Survival, need to be used.

? **TROUBLESHOOTING**

- 97 (Optional) Perform a colony PCR with primers LM73 and SS144 (Table 1) following the PCR protocol and program from Step 10. The expected amplicon has a size of 602 bp (SS144 binds in the destination vector backbone; therefore the distance between LM73 and SS144 is always the same, independent of the target site).

- 98 Inoculate two cultures in 5 ml LB medium containing 100 mg/L spectinomycin with one positive clone each. Incubate in a shaking incubator at 37 °C at 200 rpm overnight.

- 99 Purify plasmid DNA using the Plasmid Miniprep Kit I according to the manufacturer's instructions.

■ **PAUSE POINT** Plasmids can be stored at –20 °C for years.

- 100 Sequence the ligated vector with SS144 (Table 1).

- 101 Perform Gateway reaction according to Step 18 using the ligated vector from Step 99 as a backbone and the second entry vector from Step 16 that carries the desired second protospacer sequence.

■ **PAUSE POINT** LR reaction can be stored at –20 °C for a few weeks.

- 102 Transform *E. coli* NEB 5-alpha with the whole Gateway reaction. Follow transformation steps from Step 9. Plate 100 μl on LB plates containing 100 mg/L spectinomycin. Incubate at 37 °C overnight.

? **TROUBLESHOOTING**

- 103 Purify plasmid DNA using the Plasmid Miniprep Kit I according to the manufacturer's instructions.

- 104 Sequence the ligated vector with the primer SS144 (Table 1).

■ **PAUSE POINT** Plasmids can be stored at –20 °C for years.

**Transformation of *Agrobacterium* with destination vector (Timing 3 d), cultivation (Timing 4–5 weeks), floral dip transformation (Timing 4 d) and propagation of *A. thaliana* (Timing 4–5 weeks), and selection and DNA extraction from primary transformants (Timing 16 d)**

- 105 Follow Steps 24–37 to transform *A. tumefaciens* GV3101 with the vector from Step 103, then Steps 38–61, using twice the amount (800 ml) of infiltration medium to transform 20 plants and using 10 plates and twice the amount of seeds to select 100 primary transformants.

- 106 Extract the DNA of the primary transformants following ref. <sup>36</sup>.

▲ **CRITICAL STEP** For ddPCR analysis, it is necessary to use an extraction method that extracts DNA with higher yield and purity than the method described in Steps 62–70.

**ddPCR analysis ● Timing 2 d**

- 107 Prepare the mastermix for a probe-based duplex assay as follows using primers and probes as detailed in Table 1:

Component	Amount ( $\mu\text{l}$ )	Final concentration
ddH <sub>2</sub> O	5.1	
2× ddPCR Probes Supermix	11	1×
Forward primer (junction; 100 $\mu\text{M}$ )	0.2	0.91 $\mu\text{M}$
Reverse primer (junction; 100 $\mu\text{M}$ )	0.2	0.91 $\mu\text{M}$
Probe (junction; 10 $\mu\text{M}$ )	0.55	0.25 $\mu\text{M}$
Forward primer (normalization; 100 $\mu\text{M}$ )	0.2	0.91 $\mu\text{M}$
Reverse primer (normalization; 100 $\mu\text{M}$ )	0.2	0.91 $\mu\text{M}$
Probe (normalization; 10 $\mu\text{M}$ )	0.55	0.25 $\mu\text{M}$
Restriction enzyme (diluted with Diluent B to 4 U/ $\mu\text{l}$ )	1	0.18 U/ $\mu\text{l}$
Template (4 ng/ $\mu\text{l}$ )	3	0.55 ng/ $\mu\text{l}$

108 Repeat Steps 85 and 86.

109 Run the ddPCR as follows:

Cycle	Denature	Anneal	Extend/stabilize droplets	Hold
1	95 °C, 10 min; 2 °C/s			
2–40	94 °C, 30 s; 2 °C/s	T <sub>A</sub> , 1 min; 2 °C/s	72° C, 2 min; 2 °C/s	
41			98 °C, 10 min; 2 °C/s	
42				12 °C, hold; 2 °C/s

110 Repeat Step 88.

111 To analyze the experiment, use the QuantaSoft Analysis Pro Software according to the manual's instructions.

### Stages 2–6: Cas experiment with selected protospacers Stage 2: cloning of selected protospacers into EC promoter plasmid ● Timing 4–5 d

112 Follow Steps 92–104, but use pDe-Sa-Cas9 EC instead to generate heritable chromosomal rearrangements.

### Stage 2: transformation of *Agrobacterium* with destination vector (Timing 3 d), cultivation (Timing 4–5 weeks), floral dip transformation (Timing 4 d) and propagation of *A. thaliana* (Timing 4–5 weeks) and primary transformant selection (Timing 16 d)

113 Follow transformation, cultivation, floral dip, propagation and primary transformant selection protocols from Steps 24–61, using twice the amount (800 ml) of infiltration medium to transform 20 plants, and using GM plates containing 6 mg/L PPT and 500 mg/L cefotaxime for primary transformant selection instead.

114 For propagation, select more primary transformants (40–80) than for the cutting efficiency test.

#### ? TROUBLESHOOTING

### Stage 2: propagation ● Timing 6–7 weeks

115 Transfer primary transformants to 5 × 5 cm pots filled with soil (substrate and vermiculite in ratio 1:1).

116 Propagate for 6–7 weeks until seed set.

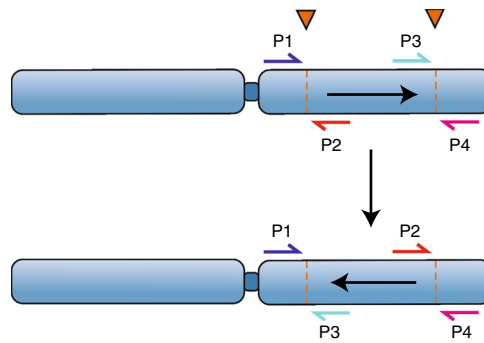
117 Harvest 200–400 ripe seeds of each individual propagated plant.

■ **PAUSE POINT** Seeds can be stored in dark and dry conditions at RT for years.

### Stages 3 and 4: T2 screening and (optional) analysis of T-DNA segregation

#### ● Timing 2–3 weeks plus 2 weeks for T-DNA segregation and 6–7 weeks for propagation

118 *Stage 3: bulk T2 screening.* Sterilize seeds according to Steps 58–61 (sterilize ~50 seeds per plate), and use 0.5 ml of 4% (vol/vol) sodium hypochlorite and 0.5 ml of sterile ddH<sub>2</sub>O for the wash steps and for stratification. Place at 4 °C for stratification overnight. Plate 50 seeds of each T1 line on a 92 × 16 mm GM plate without ventilation cams. Grow for 2 weeks in a growth chamber under 16 h light/8 h dark conditions at 22 °C.



**Fig. 5 | Primer combinations for PCR screenings, exemplary shown for an inversion event.** After induction of the inversion, the primer combinations spanning the two cut sites (cut site 1: P1 and P2, cut site 2: P3 and P4, shown on the top image of the original, pre-inversion chromosome) are used in new combinations to amplify the newly formed junctions (junction 1: P1 and P3, cut site 2: P2 and P4, see bottom image). Orange arrowheads indicate CRISPR-Cas-induced DSBs.

- 119 To reduce screening time, implement a bulk screening method<sup>4</sup> using the DNA extraction method from Steps 62–70. However, in contrast to the single plant DNA extraction, cut off one leaf from 40 plants per plate and place all into one single microcentrifuge tube. Resuspend pellets in 100  $\mu$ l TE buffer (pH 8.0).
- 120 To screen for the presence of the desired mutation, use the primers from Step 71 (Table 1) in new combinations (P1/P3 and P2/P4 as depicted in Fig. 5) to screen for the newly generated junctions. Perform a DreamTaq Polymerase PCR reaction to screen for the newly formed junctions:

Component	Amount ( $\mu$ l)	Final concentration
10 $\times$ DreamTaq Buffer	2.5	1 $\times$
dNTPs (10 $\mu$ M)	0.5	0.2 $\mu$ M
Forward primer (50 pmol/ $\mu$ l)	0.25	0.5 pmol/ $\mu$ l
Reverse primer (50 pmol/ $\mu$ l)	0.25	0.5 pmol/ $\mu$ l
DreamTaq polymerase (5 U/ $\mu$ l)	0.13	0.026 U/ $\mu$ l
Template (DNA samples from Step 119 and Col-0)	2	
ddH <sub>2</sub> O	19.37	
Total	25	

Use the PCR program below. Annealing temperature corresponds to the  $T_m$  of the used primers. Calculate extension time based on the polymerase’s ability to extend 1–2 kb/min (for further information, see product information).

Cycle	Denature	Anneal	Extend	Hold
1	95 $^{\circ}$ C, 3 min			
2–30	95 $^{\circ}$ C, 30 s	x $^{\circ}$ C, 30 s	72 $^{\circ}$ C, x s	
31			72 $^{\circ}$ C, 2 min	
32				12 $^{\circ}$ C, hold

**■ PAUSE POINT** PCR reaction can be stored for weeks at 4  $^{\circ}$ C or –20  $^{\circ}$ C.

- 121 Mix PCR reaction with gel loading dye, and load 15  $\mu$ l on 0.9% (wt/vol) SB agarose gel containing 1% (wt/vol) ethidium bromide in 1 $\times$  SB buffer and run for 20–30 min at 200 V. The T2 pools that show amplicons of the expected size in the bulk screening are identified as positive.
- 122 *Stage 4: individual T2 screening.* Extract DNA from individual plants of the respective lines identified in Step 121 to find individual plants carrying the rearrangement by repeating Steps 119–121

**? TROUBLESHOOTING**

- 123 If plants are identified that carry the mutation, repeat the PCR with the single plants in twice the volume and test for wild-type bands.

- 124 Add gel loading dye to the PCR reaction and mix. Load 3  $\mu$ l of the PCR product on a 0.9% (wt/vol) SB agarose gel containing 1% (wt/vol) ethidium bromide in 1 $\times$  SB buffer, and run for 20–30 min at 200 V. If amplicons of the expected size are displayed on the gel, purify remaining PCR product. Sequence PCR product with the respective forward and reverse primers.  
**■ PAUSE POINT** Purified PCR product can be stored for weeks at  $-20^{\circ}\text{C}$ .
- 125 (Optional) *T-DNA segregation*. Sterilize seeds according to Step 118, and sow on 92  $\times$  16 mm GM plates without ventilation cams containing 6 mg/L PPT. Grow for 2 weeks in a growth chamber under 16 h light/8 h dark conditions at 22  $^{\circ}\text{C}$ .
- 126 Count dead plants (growth stop after two-leaf stage), nongerminated seeds and viable plants, and use the chi-squared test with the critical value  $\chi^2$  (1; 0.95) to determine whether T-DNA segregation followed Mendel's laws of segregation. Preferably, propagate plants following Mendel's laws of segregation as it is likely that in these lines there will be a sufficient number of homozygous plants for propagation in the T3 generation.
- 127 *Propagation*. Transfer plants carrying the rearrangement, as confirmed in Steps 122–124, to 5  $\times$  5 cm pots filled with soil (substrate and vermiculite in ratio 1:1).
- 128 Grow under 16 h light/8 h dark conditions at 22  $^{\circ}\text{C}$  in the greenhouse until seed set (~6–7 weeks).
- 129 Harvest 200 ripe seeds of each individual plant.  
**■ PAUSE POINT** Seeds can be stored in dark and dry conditions at RT for years.

### Stage 5: T3 screening and T-DNA screening ● Timing 2–3 weeks

- 130 Repeat the sterilization and screening process from Steps 118–121 for the propagated T2 lines from Step 129.
- 131 In addition, screen for wild-type junctions to determine the genotype of the screened plants. Use the primers from Step 71 (Table 1) in the wild-type combination (P1/P2 and P3/P4, as depicted in Fig. 5). Perform a DreamTaq Polymerase PCR reaction and gel electrophoresis according to Steps 120–121.
- 132 Screen for the presence of the T-DNA with primers CS331 and JS252 (Table 1) using the standard PCR protocol and program from Steps 120–121.

### Stage 6: propagation ● Timing 6–7 weeks

- 133 Transfer homozygous plants which do not contain the T-DNA anymore (if present) to 5  $\times$  5 cm pots filled with soil (substrate and vermiculite in ratio 1:1).
- 134 Grow under 16 h light/8 h dark conditions at 22  $^{\circ}\text{C}$  in the greenhouse for 6–7 weeks until seed set.
- 135 Harvest each plant separately, and obtain ~200 ripe seeds per plant. Harvested seeds carry the mutation homozygously.
- 136 If plants were propagated that still contained the T-DNA, T-DNA screening and propagation can be repeated until plants do not carry the Cas construct anymore.
- 137 Seeds can be stored in dark and dry conditions at RT for years.

## Troubleshooting

Troubleshooting advice can be found in Table 2.

**Table 2 | Troubleshooting table**

Step	Problem	Possible reason	Solution
9	No colonies	Restriction enzyme did not cleave	Adjust incubation time Increase digestion volume
	No positive colonies	Wrong selection medium Ligation failed	Use LB plates containing the appropriate amount of ampicillin Increase ligation volume Adjust incubation time and temperature
19	No colonies	Gateway reaction failed	Increase incubation time
	No positive colonies	Used ccdB-resistant <i>E. coli</i> strain	Use ccdB-sensitive <i>E. coli</i> strain

Table continued

Table 2 (continued)

Step	Problem	Possible reason	Solution
31	No colonies	Used <i>A. tumefaciens</i> with low transformation efficiency Wrong selection medium	Use <i>A. tumefaciens</i> with high transformation efficiency Use YEB plates containing the appropriate amount of rifampicin, gentamycin and spectinomycin
61, 114	No primary transformants	Wrong selection medium <i>A. thaliana</i> transformation failed	Use GM plates containing PPT and cefotaxime Follow the protocol of ref. <sup>32</sup>
81	No cleavage activity	Poor protospacer design	Ensure that protospacer does not contain PAM, but that the target site is accompanied by the PAM
96	Low cleavage activity	Weak protospacer	Choose another target sequence
	No colonies	Ligation failed	Increase ligation volume Adjust incubation time and temperature
102	No positive colonies	Used ccdB-sensitive <i>E. coli</i> strain	Use ccdB-resistant <i>E. coli</i> strain
	No colonies	Gateway reaction failed	Increase incubation time
122	No positive colonies	Used ccdB-resistant <i>E. coli</i> strain	Use ccdB-sensitive <i>E. coli</i> strain
	No heritable events	Used wrong promoter	Use egg-cell-specific promoter
		Did not screen enough plants Wrong primers	Screen up to 80 lines Check primer design and combination

## Timing

### Stage 1

Steps 1–2, design sgRNAs for target gene: 1 d  
 Steps 3–17, construction of entry vector: 3 d  
 Steps 18–23, construction of destination vector: 3 d  
 Steps 24–37, transformation of *Agrobacterium*: 3 d  
 Steps 38–40, cultivation of *A. thaliana*: 4–5 weeks  
 Steps 41–56, floral dip transformation: 4 d  
 Step 57, propagation of *A. thaliana*: 4–5 weeks  
 Steps 58–61, primary transformant selection: 16 d  
 Steps 62–70, DNA extraction: 3 h to 1 d  
 Steps 71–76, preparation for TIDE analysis of mutation rates: 1 d  
 Steps 77–81, TIDE analysis: 1 d

### Stage 1: optional

Step 82, design of ddPCR assay: 1 d  
 Step 83, design of ddPCR control constructs: 4 d  
 Steps 84–91, gradient PCRs in preparation for ddPCR assay: 4 d  
 Steps 92–104, construction of two gRNA construct for ddPCR: 4–5 d  
 Steps 105 and 106, transformation of *Agrobacterium*: 3 d; cultivation of *A. thaliana*: 5 weeks; floral dip transformation: 4 d; propagation of *A. thaliana*: 4–5 weeks; and primary transformant selection: 16 d  
 Steps 107–111, ddPCR analysis: 2 d

### Stage 2

Step 112, construction of two gRNA construct for the induction of heritable chromosomal rearrangements: 4–5 d  
 Steps 113 and 114, transformation of *Agrobacterium*: 3 d; cultivation of *A. thaliana*: 5 weeks; floral dip transformation: 4 d; propagation of *A. thaliana*: 4–5 weeks; and primary transformant selection: 16 d  
 Steps 115–117, propagation of *A. thaliana*: 6–7 weeks

### Stages 3 and 4

Steps 118–126, bulk and individual T2 screening and (optional) T-DNA segregation analysis: 2–3 weeks (plus 2 weeks for T-DNA segregation)  
 Steps 127–129, propagation of *A. thaliana*: 6–7 weeks

### Stage 5

Steps 130–132, T3 screening and genotyping: 2–3 weeks



## Stage 6

Steps 133–137, propagation of *A. thaliana*: 6–7 weeks

## Anticipated results

In the case of the rknob inversion in the Col-0 background, we found that 10 out of 38 T2 lines showed PCR bands for both inversion junctions in the bulk screening, which combined the DNA of 40 individual plants per line (Table 3)<sup>5</sup>. Five of these T2 lines were further subjected to analysis of the 40 individual plants. We found 40 plants positive for both junctions among the five tested pools. In four T2 lines, 1–3 out of 40 plants carried the rearrangement. In the fifth line, 34 out of 40 plants were found to carry both junctions, suggesting an event occurred early in the T1 generation. From each identified line, plants were propagated. In the following generation, multiple lines, each homozygous for the inversion, were detected. In total, seven plants homozygous for the inversion were identified. Most of the plants and junctions (10 out of 14 junctions) carried a perfect inversion in terms of perfect re-ligation at the junctions.

In the case of the reciprocal translocation between chromosome I and II in the Col-0 background, we were able to identify 3 out of 40 T2 lines that showed PCR bands for both of the translocation junctions in the bulk screening (Table 4)<sup>6</sup>. Again, we found plants positive for both junctions among each of the tested pools. In total, four individual plants harboring the reciprocal translocation were identified and further propagated. In the following generation, 20–30% of the plants were homozygous for the reciprocal translocation. This high number can be explained by the inability of unbalanced gametes to contribute to the germline. Three of the four lines carried perfect translocations in terms of perfect re-ligation at both of the junctions. The frequencies obtained for the second translocation were lower (0.03% versus 0.1%) because of lower mutation rates at the respective target sites. It was demonstrated that performing the experiments in a *ku70-1* T-DNA insertion line background generates higher mutation frequencies (Table 4)<sup>4,6</sup>. However, this goes along with an increased number of InDels at the junctions.

**Table 3 | Overview of induced knob inversions detected in the T2 generation in Col-0 background**

Background	Positive T2 pools	Frequency of positive T2 pools	Number of T2 pools subjected to analysis of individual plants	Positive individually screened plants	Frequency of positive individually screened T2 plants
Col-0	10/38	26.3%	5	Pool 1: 1, Pool 2: 1, Pool 3: 1, Pool 4: 3, Pool 5: 34	Pool 1: 2.5%, Pool 2: 2.5%, Pool 3: 2.5%, Pool 4: 7.5%, Pool 5: 85.0%

Positive T2 lines were determined by PCR-based bulk screening (pools of 40 plants per line). Table adapted with permission from ref. <sup>5</sup>, Springer Nature Limited.

**Table 4 | Overview of induced translocations detected in the T2 generation in Col-0 and *ku70* T-DNA insertion line background**

Background	Positive T2 pools	Frequency of positive T2 pools	Number of T2 pools subjected to analysis of individual plants	Positive individually screened plants	Frequency of positive individually screened T2 plants
Col-0	3/40	7.5%	3	Pool 1: 1, Pool 2: 1, Pool 3: 2	Pool 1: 2.5%, Pool 2: 2.5%, Pool 3: 5.0%
<i>ku70</i>	4/20	20.0%	4	Pool 1: 1, Pool 2: 3, Pool 3: 1, Pool 4: 3	Pool 1: 2.5%, Pool 2: 7.5%, Pool 3: 2.5%, Pool 4: 7.5%

Positive T2 lines were detected by PCR-based bulk screening (two pools of 40 plants per line). Table adapted with permission from ref. <sup>6</sup>, Springer Nature Limited.

### Data availability

The data shown in Tables 3 and 4 are available in the supporting primary research papers (refs. <sup>5,6</sup>, respectively). Figure 4 represents data that have not been previously published and are provided as Supplementary Data 1.

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### Author contributions

All authors wrote the manuscript. M.R., P.S. and R.W. designed and created the figures.

### Competing interests

The authors declare no competing interests.

### Additional information

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