Massive Crossover Suppression by CRISPR-Cas-mediated Plant Chromosome Engineering

Michelle Rönspies

Karlsruhe Institute of Technology <https://orcid.org/0000-0003-2659-6829>

Carla Schmidt

DKFZ Heidelberg <https://orcid.org/0000-0002-4715-4985>

Michal Lieberman-Lazarovich

Agricultural Research Organization Volcani Center <https://orcid.org/0000-0002-1776-8257>

Patrick Schindele

Karlsruhe Institute of Technology

Andreas Houben

Institute of Plant Genetics and Crop Plant Research <https://orcid.org/0000-0003-3419-239X>

Holger Puchta (✉ holger.puchta@kit.edu)

Karlsruhe Institute of Technology <https://orcid.org/0000-0003-1073-8546>

Letter

Keywords:

Posted Date: January 20th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1257133/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Nature Plants on September 15th, 2022.
See the published version at <https://doi.org/10.1038/s41477-022-01238-3>.

Abstract

Recent studies have demonstrated that not only genes, but also entire chromosomes can be engineered using CRISPR-Cas9 ^{1–5}. A major objective of applying chromosome restructuring (CR) in plant breeding is the manipulation of genetic exchange ⁶. Here we show that meiotic recombination can be suppressed in nearly the entire chromosome using CR. We were able to induce a heritable inversion of a more than 17 Mb-long chromosome fragment, which contained the centromere and covered most of chromosome 2 of the *Arabidopsis* ecotype Col-0. Only the 2 and 0.5 Mb-long telomeric ends remained in their original orientation. In SNP marker analysis with the offspring of crosses with the ecotype Ler-1, we detected a massive reduction of crossovers (CO) within the inverted chromosome region, coupled with a shift of CO to the telomeric ends. Interestingly, the few genetic exchanges detected within the inversion all originated from double CO. This not only indicates that heritable genetic exchange can occur by interstitial chromosome pairing, but also that it is restricted to the production of viable progeny.

Main

CRISPR-Cas-based gene editing has revolutionized plant biology and breeding ⁷. More and more tools are being developed to finetune both single and multiple gene modifications ^{8–10}. However, changing the order of genes on a chromosome also adds a new level of trait control: the breakage of genetic linkages ¹¹. To combine attractive traits in a single cultivar, breeders rely on natural crossovers (CO) between parental homologous chromosomes by meiotic recombination ¹⁸. However, chromosomal rearrangements, such as inversions, modulate the recombination landscape along a chromosome by suppressing CO in the rearranged area ¹⁹. Pan-genome studies have found that chromosomal rearrangements are prevalent in many crop species and play an important role in domestication ^{4,12–17}. Despite their seemingly deleterious effects inversions can also result in positive effects, such as the protection of favorable allelic combinations by preventing recombination ²⁰. Therefore, the targeted induction of chromosomal rearrangements by CRISPR-Cas has the potential to change meiotic recombination patterns. By reverting a 1.1 Mb-sized naturally-derived inversion on chromosome 4 in the *Arabidopsis thaliana* ecotype Col-0, we could show that CO can be induced in a previously recombination-cold region ¹.

In this study, we aimed to investigate whether we could exclude a significant part of the *Arabidopsis* genome from genetic exchange by CRISPR-Cas-mediated chromosome engineering. To exclude as much genetic information as possible from meiotic recombination, we decided to invert nearly an entire chromosome and investigated the effect of the inversion on CO frequency and distribution. To achieve this goal, we used a well-established protocol for obtaining rare CR events in *Arabidopsis* ²¹. By combining the *S. aureus* ortholog of Cas9 ²² with an egg cell-specific promoter, two double strand breaks were induced in close proximity to the telomeric ends of chromosome 2 to invert the interjacent fragment with a size of 17.1 Mb. This left only a 2 Mb- and a 0.5 Mb-long telomeric end in their original orientation (Fig. 1a). The two selected guide sequences had both been used for the induction of inversions and

translocations successfully before^{23,24}. The selection and detection of the inversion was performed as previously described¹. Out of the 40 screened T2 pools, one T2 pool was identified as positive for the inversion. Subsequently, the 40 descendants of the positive line were screened individually and one plant of the respective line was found to carry the inversion. The inversion junctions were amplified by PCR and sequenced to analyze the molecular nature of the junctions (Fig. 1b). The sequencing data revealed that the DNA ends were ligated precisely without any sequence loss or gain. To determine the genotype, the plant was also screened for the wildtype (WT) junctions and was found to carry the inversion hemizygotously. The plant was grown until seed set and propagated. For subsequent experiments, T3 seeds were harvested and grown for 2 weeks on germination medium without selection and their genotype was determined by PCR screenings for the WT and inversion junctions. The phenotype of homozygous, hemizygous and WT plants did not differ from each other (Supplemental Fig. 1). The plants were also tested for segregation based on Mendel's laws of segregation by using a chi-squared test with the critical value χ^2 (1; 0.95). Mendelian segregation was confirmed (Supplemental Table 1). On the cytological level, the hemizygous state of the chromosome inversion was confirmed by FISH. To obtain purely hemizygous seeds for the analysis, the homozygous inversion line was back-crossed with Col-0. Metaphase I meiocytes of the resulting F1 plants were prepared and BAC clones (Supplemental Table 2), which spanned the two CRISPR-Cas9 cleavage sites, were chosen for red and green fluorescent labeling. In case of an inversion, both probes should be detected in close proximity to each other. In WT, they should be located apart from each other. Indeed, two separate signals were detected in WT (Fig. 1c). In the hemizygous sample, the WT signal pattern, as well as the inversion signal pattern were detected (Fig. 1c). Both newly-formed junctions can be identified by adjacent red and green fluorescence signals. Interestingly, in Figure 1c, the meiotic pairing of the chromosome carrying the inversion and the WT chromosome via the telomeric ends of the shorter arm of chromosome 2 is clearly visible.

To assess the effect of the chromosomal rearrangement on fertility, a fertility assay was performed with homozygous, hemizygous and WT plants by counting the seed number of 10 siliques from 12 plants per genotype. We found that the seed number per silique was decreased by 1/3 in the hemizygous plants compared to homozygous and WT plants (Fig. 1d). Additionally, we observed empty spaces in place of seeds in several siliques of the hemizygous plants (Supplemental Fig. 2). The seed number did not differ significantly between homozygous and WT plants.

Next, we investigated the effect of inverting approximately nine-tenths of the chromosome on the recombination frequency on chromosome 2 and, for purposes of control and comparison, on chromosome 3. The homozygous inversion line was crossed with Ler-1. F1 seeds were subsequently harvested. Then, they were propagated to obtain F2 seeds. The recombination frequency was determined by analyzing 400 plants which were grown from F2 seeds. As a control, 400 plants of the progeny of crosses between Col-0 and Ler-1 were analyzed. Using a TaqMan SNP genotyping assay, we selected 11 SNP (single-nucleotide polymorphism) markers on chromosome 2 and 9 SNP markers on chromosome 3 to detect marker changes between Col-0 and Ler-1 alleles (Supplemental Table 3). The analyzed plants were diploid; therefore, the marker analysis sums up the genetic state of two individual chromosomes

that both underwent meiosis independently. Thus, we first analyzed the presence or absence of marker changes in each sample. In the offspring of the *Inversion x Ler-1* line, we detected a massive reduction of marker changes within the inverted area compared to the Col-0 x Ler-1 control. We detected marker changes in 16 samples within the inverted region, compared to 55 in the Col-0 x Ler-1 control line in the same area. This corresponds to a reduction of recombination by roughly 71 % in the *Inversion x Ler-1* line (Fig. 2a). In total, we detected marker changes in 49 out of 400 samples on chromosome 2. In the remaining 351 samples we detected no recombination. We assume that, in most of these cases, the CO required for chromosome segregation occurred at one of the subtelomeric ends outside of our tested marker intervals: 1.8 Mb on the 5' telomeric end and 161 kb on the 3' telomeric end were not covered by our marker intervals. By comparison, in the offspring of the *Col-0 x Ler-1* control, we detected marker changes in 299 out of 400 samples. No recombination was detected in 101 samples, which again points to CO occurring in the telomeric regions not covered by our marker intervals. Notably, this number is more than three times lower than in the *Inversion x Ler-1* line. In contrast to chromosome 2, no notable difference in CO distribution between the two crosses was detected on chromosome 3 in the same plants.

Subsequently, by analyzing the marker change patterns in more detail (Supplemental Fig. 2b,c), we deduced the genetic state of the individual chromosomes from the diploid genomes. In the *Inversion x Ler-1* line, of the 49 samples in which marker changes were detected, 32 marker changes originated from single recombination events, due to a single CO on one chromosome. Another sample showed a marker change at both telomeric ends on one chromosome (Fig. 2b). All single CO were located outside of the inversion in the unchanged subtelomeric ends: 11 were detected at the 5' telomeric end and 21 at the 3' telomeric end. Interestingly, within the inversion, no single CO was detected. Among 15 samples, we found marker changes originating from a double CO in one of the chromosomes (Fig. 2c). Accordingly, within the inversion, only double CO, but not single CO, can lead to the formation of functional and complete chromosomes in this setup (Fig. 3). In another sample, we detected 3 CO, a double CO within the inversion combined with a marker change at the 3' telomeric end outside of the inversion (Fig. 2c). This conformation likely originated from the combination of a gamete with single CO at the subtelomeric end and a gamete with a double CO located in the inverted region. Out of the 299 samples with detected marker changes in the control line Col-0 x Ler-1, 225 marker changes originated from single CO events: 187 marker changes originated from a single CO on one chromosome (Supplemental Fig. 3a), 30 marker changes originated from two different single CO, one on each chromosome (Supplemental Fig. 3b), and 8 marker changes originated from an identical single CO on both chromosomes (Supplemental Fig. 3c). A double CO on one chromosome was found in 59 samples (Supplemental Fig. 3 d), whereas in another sample, 3 CO were detected (a double CO on one chromosome and a single CO on the second chromosome; Supplemental Fig. 3e). 14 samples showed marker changes originating from multiple CO events that could not be clearly assigned to either of the two chromosomes (Supplemental Fig. 3f). We analyzed the distribution of all double CO along the chromosome in both lines and found that all double CO events in the inversion line were located on the restructured long 5' chromosome arm. Most of the double CO events (56) in the control were located on the long 3' chromosome arm, one was located on the short arm and 3 events were spanning both chromosome arms. The average double CO sizes ranged

between 1.3 and 7.9 Mb in the inversion line and 1.3 to 15.5 Mb in the control (Supplemental Fig. 4). The average double CO length was with 4.5 Mb significantly shorter in the inversion line, compared to 7.1 Mb in the control.

Applying our CRISPR-Cas-based strategy for inverting chromosomal regions²⁴, we were able to invert almost nine-tenths of chromosome 2 in *Arabidopsis* which led to a near-complete exclusion of more than one-tenth of the *Arabidopsis* genome from genetic exchange. In its hemizygous state, the inversion led to a significant reduction of viable seeds, by about one third, whereas the homozygous mutant was fully fertile. The simplest explanation is that in about every third meiosis the presence of the inversion hindered the formation of viable gametes. It is known that telomeres play an important role in homolog pairing in *Arabidopsis*: During prophase I, the telomeres cluster at the nuclear membrane, forming the so-called telomere bouquet, which has been observed in various organisms²⁵. In *Arabidopsis*, the telomeres instead form a nucleolus-associated cluster²⁶. The bouquet presumably leads to a co-alignment of the homologs and confines the chromosomes to a limited space, promoting the search for homology²⁷. It has been proposed that centromere pairing during early prophase I may play a role in homolog pairing, which has been reported in many species²⁵. However, in *Arabidopsis*, centromere pairing starts first in zygotene and likely plays some other role than promoting homolog pairing²⁵. In line with this assumption, we did indeed detect more CO in the terminal regions of the chromosome carrying the inversion than in the WT chromosome. Our analysis probably strongly underestimated the number of CO in the subtelomeric regions, as a quarter of all samples in WT showed no marker exchange, which indicates that the CO required for segregation occurred outside of our tested marker intervals, close to the telomers. In the case of the inversion line, this number increased by more than threefold, pointing to a shift of CO to the telomere ends as a consequence of the inversion. Thus, within the chromosome body, recombination is drastically reduced, but it is not completely abolished. Indeed, in less than 4% of the cases, double CO could be detected within the inverted region, indicating that meiotic pairing and recombination can also occur within the inverted region. The reduced fertility of the hemizygous line indicates that this might occur even more frequently. However, as shown in Figure 3, if a single CO is induced within the inversion, the resulting restructured chromosome carries two identical chromosomal ends, which results in a massive loss of genetic information and non-viable progeny.

Our results have important consequences for plant breeding, as they demonstrate that nearly the entire chromosome can be excluded from genetic exchange. We could show that chromosome engineering cannot only be used to restore recombination in a previously CO-suppressed region or to break genetic linkages, but it can also be a valuable tool for breeders in excluding favorable allelic combinations from recombination, thereby stabilizing genetic linkages.

Declarations

Acknowledgements

We thank Julia Baumann, Nadine Schäfter and Katrin Kumke for their excellent technical assistance and Daniel Donahey for proofreading the manuscript. This research was funded by the European Research Council (ERC; Grant number ERC-2016-AdG_741306 CRISBREED) to H.P.

Author contributions

M.R., C.S. and H.P. designed and planned the experiments, M.R., C.S, M.LL. and A.H. carried out the experiments, M.R., C.S., P.S, A.H. and H.P. analyzed data, M.R. and P.S. created the figures and M.R., P.S., A.H. and H.P. wrote the paper.

Competing interests

The authors declare no competing interests.

References

1. Schmidt, C. *et al.* Changing local recombination patterns in Arabidopsis by CRISPR/Cas mediated chromosome engineering. *Nature Communications* **11**, 4418; 10.1038/s41467-020-18277-z (2020).
2. Beying, N., Schmidt, C., Pacher, M., Houben, A. & Puchta, H. CRISPR-Cas9-mediated induction of heritable chromosomal translocations in Arabidopsis. *Nature plants* **6**, 638–645; 10.1038/s41477-020-0663-x (2020).
3. Lu, Y. *et al.* A donor-DNA-free CRISPR/Cas-based approach to gene knock-up in rice. *Nature plants* **7**, 1445–1452; 10.1038/s41477-021-01019-4 (2021).
4. Schwartz, C. *et al.* CRISPR-Cas9-mediated 75.5-Mb inversion in maize. *Nature plants* **6**, 1427–1431; 10.1038/s41477-020-00817-6 (2020).
5. Filler Hayut, S., Melamed Bessudo, C. & Levy, A. A. Targeted recombination between homologous chromosomes for precise breeding in tomato. *Nature Communications* **8**, 15605; 10.1038/ncomms15605 (2017).
6. Rönspies, M., Dorn, A., Schindele, P. & Puchta, H. CRISPR-Cas-mediated chromosome engineering for crop improvement and synthetic biology. *Nature plants* **7**, 566–573; 10.1038/s41477-021-00910-4 (2021).
7. Gao, C. Genome engineering for crop improvement and future agriculture. *Cell* **184**, 1621–1635; 10.1016/j.cell.2021.01.005 (2021).
8. Molla, K. A., Qi, Y., Karmakar, S. & Baig, M. J. Base Editing Landscape Extends to Perform Transversion Mutation. *Trends in genetics : TIG* **36**, 899–901; 10.1016/j.tig.2020.09.001 (2020).

9. Atkins, P. A. & Voytas, D. F. Overcoming bottlenecks in plant gene editing. *Current opinion in plant biology* **54**, 79–84; 10.1016/j.pbi.2020.01.002 (2020).
10. Schindele, A., Dorn, A. & Puchta, H. CRISPR/Cas brings plant biology and breeding into the fast lane. *Current opinion in biotechnology* **61**, 7–14; 10.1016/j.copbio.2019.08.006 (2020).
11. Lee, K. & Wang, K. Level up to chromosome restructuring. *Nature plants* **6**, 600–601; 10.1038/s41477-020-0669-4 (2020).
12. Walkowiak, S. *et al.* Multiple wheat genomes reveal global variation in modern breeding. *Nature* **588**, 277–283; 10.1038/s41586-020-2961-x (2020).
13. Jayakodi, M. *et al.* The barley pan-genome reveals the hidden legacy of mutation breeding. *Nature* **588**, 284–289; 10.1038/s41586-020-2947-8 (2020).
14. Crow, T. *et al.* Gene regulatory effects of a large chromosomal inversion in highland maize. *PLoS Genet* **16**, e1009213; 10.1371/journal.pgen.1009213 (2020).
15. Liu, Y. *et al.* Pan-Genome of Wild and Cultivated Soybeans. *Cell* **182**, 162-176.e13; 10.1016/j.cell.2020.05.023 (2020).
16. Alonge, M. *et al.* Major Impacts of Widespread Structural Variation on Gene Expression and Crop Improvement in Tomato. *Cell* **182**, 145-161.e23; 10.1016/j.cell.2020.05.021 (2020).
17. Qin, P. *et al.* Pan-genome analysis of 33 genetically diverse rice accessions reveals hidden genomic variations. *Cell* **184**, 3542-3558.e16; 10.1016/j.cell.2021.04.046 (2021).
18. Henderson, I. R. & Bomblies, K. Evolution and Plasticity of Genome-Wide Meiotic Recombination Rates. *Annual review of genetics* **55**, 23–43; 10.1146/annurev-genet-021721-033821 (2021).
19. Crown, K. N., Miller, D. E., Sekelsky, J. & Hawley, R. S. Local Inversion Heterozygosity Alters Recombination throughout the Genome. *Current biology : CB* **28**, 2984-2990.e3; 10.1016/j.cub.2018.07.004 (2018).
20. Fransz, P. *et al.* Molecular, genetic and evolutionary analysis of a paracentric inversion in *Arabidopsis thaliana*. *Plant J* **88**, 159–178; 10.1111/tpj.13262 (2016).
21. Rönspies, M., Schindele, P., Wetzel, R. & Puchta, H. CRISPR-Cas9-mediated chromosome engineering in *Arabidopsis thaliana*. *Nature Protocols* **Accepted article** (2022).
22. Steinert, J., Schiml, S., Fauser, F. & Puchta, H. Highly efficient heritable plant genome engineering using Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus*. *Plant J* **84**, 1295–1305; 10.1111/tpj.13078 (2015).

23. Beying, N., Schmidt, C., Pacher, M., Houben, A. & Puchta, H. CRISPR–Cas9-mediated induction of heritable chromosomal translocations in *Arabidopsis*. *Nature plants* **6**, 638–645; 10.1038/s41477-020-0663-x (2020).
24. Schmidt, C., Pacher, M. & Puchta, H. Efficient induction of heritable inversions in plant genomes using the CRISPR/Cas system. *Plant J* **98**, 577–589; 10.1111/tpj.14322 (2019).
25. Osman, K., Higgins, J. D., Sanchez-Moran, E., Armstrong, S. J. & Franklin, F. C. H. Pathways to meiotic recombination in *Arabidopsis thaliana*. *New Phytologist* **190**, 523–544; 10.1111/j.1469-8137.2011.03665.x (2011).
26. Armstrong, S. J., Franklin, F. C. & Jones, G. H. Nucleolus-associated telomere clustering and pairing precede meiotic chromosome synapsis in *Arabidopsis thaliana*. *Journal of cell science* **114**, 4207–4217 (2001).
27. Scherthan, H. A bouquet makes ends meet. *Nature Reviews Molecular Cell Biology* **2**, 621–627; 10.1038/35085086 (2001).
28. Steinert, J., Schmidt, C. & Puchta, H. Use of the Cas9 Orthologs from *Streptococcus thermophilus* and *Staphylococcus aureus* for Non-Homologous End-Joining Mediated Site-Specific Mutagenesis in *Arabidopsis thaliana*. *Methods in molecular biology (Clifton, N.J.)* **1669**, 365–376; 10.1007/978-1-4939-7286-9_27 (2017).
29. Lysak, M. A., Fransz, P. F., Ali, H. B. & Schubert, I. Chromosome painting in *Arabidopsis thaliana*. *Plant J* **28**, 689–697; 10.1046/j.1365-313x.2001.01194.x (2001).

Methods

Cloning of T-DNA constructs

For cloning of the T-DNA constructs, the plasmids pDe-Sa-Cas9 and pEn-Sa-Chimera were used^{22,28}. As described previously, the kanamycin resistance cassette of pDe-Sa-Cas9 was replaced by a bar resistance cassette and the PcUbi-promoter and Pea3A-terminator were replaced by the EC1.1-promoter and rbcS-E9 terminator to generate pDe-EC-Sa-Cas9¹. Guide sequences were cloned individually into pEn-Sa-Chimera. Via MluI restriction digest, the first gRNA cassette was integrated into pDe-EC-Sa-Cas9. The second gRNA cassette was transferred via a Gateway® LR-reaction. Spacer sequences and primers used for cloning are listed in Supplemental Table 4.

Plant cultivation and transformation

A. thaliana seeds were stratified overnight at 4 °C and transferred to soil consisting of a 1:1 mixture of Floraton 3 (Floragard) and vermiculite (2-3 mm, Deutsche Vermiculite Dämmstoff). For propagation, the plants were cultivated in the greenhouse under 16 h light/8 h dark conditions at 22 °C for 6-7 weeks until

seed set. For transformation, 4-5 week-old Col-0 plants were transformed with the CRISPR-Cas expression construct via *Agrobacterium*-mediated floral dip transformation. The transformed plants were cultivated for 4-5 weeks until seed set. For sterile plant culture, seeds were surface-sterilized with 4% sodium hypochlorite and stratified overnight at 4 °C. For transgenic plant selection, the stratified seeds were sowed on germination medium (GM; 4.9 g l⁻¹ Murashige and Skoog medium, 10 g l⁻¹ saccharose, pH 5.7 and 7.6 g l⁻¹ plant agar) with phosphinotricin and cefotaxime. T1 plants were propagated in the greenhouse and the seeds harvested. T2 seeds were stratified and sowed on germination medium (GM; 4.9 g l⁻¹ Murashige and Skoog medium, 10 g l⁻¹ saccharose, pH 5.7 and 7.6 g l⁻¹ plant agar) without further additives. The plates were placed in a growth chamber at 22 °C under 16 h light/8 h dark conditions for 2 weeks.

DNA extraction

For individual plant screenings, one leaf per plant was cut off and separately placed into a 1.5 mL reaction tube. For bulk screenings, as described previously ²⁴, one leaf of 40 plants each was cut off and combined in the same 1.5 ml reaction tube. The plant material was ground with a pestle and 500 µL extraction buffer (200 mM Tris-HCl (pH 9.0), 400 mM LiCl, 25 mM EDTA, 1% SDS, pH 9.0) was added to the tubes. The mixture was centrifuged for 5 min at 17,000 g at room temperature. 400 µL of the supernatant were transferred to a new 1.5 mL tube containing 400 µL 2-propanol. The sample was thoroughly inverted and the DNA was pelleted for 10 min at 19,500 g at room temperature. The supernatant was discarded and the pellet was dried, either for 1.5 h at 37 °C or overnight at room temperature. Afterwards, the pellets were resuspended in 50 µL (individual DNA extraction) or 100 µL (bulk DNA extraction) TE buffer (10 mM Tris-HCl (pH 9.0), 1.0 mM EDTA, pH 8.0).

Establishment of the homozygous inversion line

The detection of the heritable inversion was carried out according to our previously described bulk screening protocol ²⁴. T2 seeds were cultivated on GM medium for 2 weeks in a growth chamber. Subsequently, bulk DNA extractions were performed with 40 plants per T2 line. These T2 pools were screened for the presence of the inversion via PCR using junction-specific primers (Supplemental Table 4). Sequencing of the junctions was performed by Eurofins Genomics and the software ApE (v2.0.55) was used for alignment and analysis of Sanger sequencing data. One T2 line tested positive for the inversion. The individual plants of this line were subjected to individual DNA extraction to identify the individual plants harboring the rearrangement. The individual DNA samples were again screened by PCR. The identified positive plant was transferred to the greenhouse and grown for 6-7 weeks until seed set. In the T3 generation, the seeds were genotyped by PCR using primers specific for the WT and inversion junctions (Supplemental Table 4). The line was also tested for Mendelian segregation, based on the genotyping results, using a chi-squared test with the critical value χ^2 (1; 0.95). Homozygous plants and, as a control, Col-0 plants were crossed with the Ler-1 ecotype for subsequent determination of CO frequencies. F1 seeds were propagated in the greenhouse and ripe F2 seeds were harvested after 6-7 weeks.

Phenotypic analysis and fertility assays

Homozygous, hemizygous and WT plants, as determined by PCR-based genotyping in the T3 generation, were grown in the greenhouse for 5-6 weeks under 16 h light/8 h dark conditions at 22 °C. After representative pictures were taken for phenotype analysis, 10 mature siliques of 12 plants per line were collected and incubated overnight in 70% EtOH to determine the effects of the inversion on fertility. The number of seeds per silique was counted and pictures of the siliques were taken using a binocular microscope. Statistical significance was calculated using a one-way ANOVA analysis. Graphs were created using R Studio (Version 1.1.423) and CorelDRAW 2020 (Version 22.1.1.523).

Determination of CO frequency

The CO frequency was determined by the detection of marker changes via SNP-based genotyping using cultivar-specific probes. For chromosome 2, 11 TaqMan probes and specific primers were designed. For chromosome 3, 9 TaqMan SNP genotyping assays were designed. All probes and primers are listed in Supplemental Tables 3 and 4. The analysis was performed using a Lightcycler® 480 II (Roche) and the PerfeCTa® qPCR ToughMix® (Quantabio), according to the manufacturer's protocol, but with a total reaction volume of 10 µL. The data were analyzed with LightCycler® 480 SW 1.5. Graphs were created using Microsoft Excel 2016 and CorelDRAW 2020 (Version 22.1.1.523).

Fluorescence in situ hybridisation.

FISH analysis was performed as described previously ²⁹, using differently labeled DNA probes specific for the inversion breakpoint regions of chromosome 2. Pools of contiguous BAC clones of chromosome 2 were used to paint the neighbouring regions of the CRISPR-Cas9 cleavage sites (Supplemental Table 2). An epifluorescence microscope (BX-61, Olympus), equipped with UPlan(F), 100x/1.30 objective lens (Olympus) and a cooled black/white CCD camera (ORCA-R2, Hamamatsu) under the control of the microscope software CellSens Dimension (Olympus), was used to record the micrographs. The following fluorescence filters were used: FITC-2024B-000, Txred-4040C-000n and Sp. Aqua HC (Sembrock). Black and white pictures were pseudo-coloured with the software Photoshop 2020 (Adobe).

Figures

Figure 1

Schematic overview of the induced chromosome inversion, WT- and inversion junctions, fertility analysis and FISH analysis. **a**, Schematic overview of the induced chromosome inversion. Orange triangles and dashed lines indicate the location of the CRISPR-Cas9-induced DSB. **b**, DNA sequence of the two WT- and inversion-specific junctions. The 5' guide sequence is highlighted in blue and the corresponding PAM sequence in red. The 3' guide sequence is highlighted in yellow and the corresponding PAM sequence in

pink. The first two lines show the original WT conformation. The last two lines show the junctions after induction of the inversion in their new conformation **c**, Overview of the FISH analysis of the hemizygous inversion line. Left: Schematic overview of the fluorescently labeled BAC clones spanning the 5' (red) and 3' (green) cleavage site. Black triangles indicate the location of the DSB. The induction of the inversion leads to the presence of both fluorescence signals at both ends of the chromosome. In the hemizygous mutant, both the WT and inversion fluorescence pattern are present. Right: FISH results of the analyzed metaphase I meiocytes of WT (top) and hemizygous mutant (bottom). Two separate signals were detected in WT. In the hemizygous sample, the WT signal pattern, as well as the inversion signal pattern were detected. Both newly-formed junctions can be identified by adjacent red and green fluorescence signals. **d**, Fertility analysis of homozygous, hemizygous and WT plants. 12 homozygous, hemizygous and WT plants were randomly selected for fertility analysis (n=12). 10 siliques per plant were randomly selected and the number of seeds per silique counted. The seed number of the hemizygous plants was significantly reduced, approximately by 1/3, compared to WT and homozygous plants. The seed number of homozygous and WT plants did not differ significantly. The boxplot shows the 12 independent biological replicates for each genotype. The average of the seed number of 10 siliques was calculated and is presented in the boxplots. The middle line represents the median, box edges represent first and third quartiles and whiskers extend to the minimum and maximum, respectively. Outliers are represented as circles. The p values were calculated using a two-tailed t-test (***) $p < 0.001$; n.s. not significant). Source data are provided as Source Data file.

Source Data file. **b**, Overview of marker changes detected outside of the inverted area. These 33 marker changes all originated from single CO events in the telomeric ends. The Col-0 allele is represented by blue bars and the Ler-1 allele by red bars. **c**, Overview of marker changes detected inside of the inverted area. In 15 samples, marker changes inside the inversion were detected which all originated from double CO events. The Col-0 allele is represented by blue bars and the Ler-1 allele by red bars. One other sample showed both a double CO inside the inversion and a single CO at the 3' telomeric end. The detection of both the Col-0 and Ler-1 allele is represented by orange bars. This conformation likely resulted from the combination of a gamete with a double CO and a gamete with a single CO at the telomeric end.

Figure 3

Consequences of the occurrence of subtelomeric, single or double CO in a large chromosome inversion.

Left panel: Pairing of the homologous regions of the unchanged distal chromosome regions, followed by single or double CO in the subtelomeric regions, results in viable gametes. Middle panel: Pairing of the large homologous region within the inversion borders, followed by a single CO within the inverted area, leads to the loss of one telomeric end in both chromosomes, resulting in inviable gametes. Right panel: Pairing of the large homologous region within the inversion borders, followed by a double CO within the inverted area, leads to viable gametes as no genetic information is lost.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalInformation.docx](#)
- [Figure1dSourceData.xlsx](#)
- [Figure2aSourceData.xlsx](#)
- [SupplementalFigure4SourceData.xlsx](#)
- [rsflat.pdf](#)