

Reverse breeding in *Arabidopsis thaliana* generates homozygous parental lines from a heterozygous plant

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Traditionally, hybrid seeds are produced by crossing selected inbred lines. Here we provide a proof of concept for reverse breeding, a new approach that simplifies meiosis such that homozygous parental lines can be generated from a vigorous hybrid individual. We silenced *DMC1*, which encodes the meiotic recombination protein DISRUPTED MEIOTIC cDNA1, in hybrids of *A. thaliana*, so that non-recombined parental chromosomes segregate during meiosis. We then converted the resulting gametes into adult haploid plants, and subsequently into homozygous diploids, so that each contained half the genome of the original hybrid. From 36 homozygous lines, we selected 3 (out of 6) complementing parental pairs that allowed us to recreate the original hybrid by intercrossing. In addition, this approach resulted in a complete set of chromosome-substitution lines. Our method allows the selection of a single choice offspring from a segregating population and preservation of its heterozygous genotype by generating homozygous founder lines.

Hybrid vigor is essential to produce high-yielding varieties in many crops¹. However, a favorable heterozygous genotype cannot be stably propagated through seeds because parental chromosomes will recombine before being passed on to progeny. Recombination of alleles is influenced by two interrelated events during meiosis I: crossover recombination and the orientation of homologous chromosomes on the metaphase plate. Crossover recombination leads to new allele combinations by reciprocal exchange of chromosome segments, whereas random orientation during metaphase I generates novel combinations of parental chromosomes. If there were no crossover recombination, the only factor diversifying genetic information would be chromosome orientation, and, consequently, intact parental chromosomes would segregate to the gametes.

Because hybrids cannot be stably maintained, breeders recreate elite hybrids afresh through crossing homozygous parental lines. Such lines simultaneously provide the means to improve hybrid performance by improving its parents. The inability of breeders to easily establish breeding lines for uncharacterized heterozygotes is a major obstacle to adopting elite heterozygotes from outbreeding

(or other segregating) populations into their hybrid breeding programs. Clonal propagation (or apomixis²) allows for the preservation of the parental genotype, but prevents its further improvement through adapting parental lines.

We previously introduced an approach termed reverse breeding³ in which meiotic recombination is suppressed and gametes are directly converted into adult plants. Here we show that reverse breeding can construct homozygous parental lines that, when mated, perfectly reconstitute the selected heterozygous genotype. These homozygous parents can be propagated indefinitely and crossed at will, in contrast to a heterozygote, which will lose its desirable genotype if propagated sexually.

RESULTS

Silencing of *DMC1* suppresses crossover recombination

The first step in reverse breeding is to produce gametes from the desired heterozygote without crossover recombination. This is best achieved by dominantly suppressing one of several genes required for meiotic recombination⁴; complete knockout of a gene by a recessive mutation is not suitable for this purpose as it would reintroduce the same mutation into the reverse-breeding offspring. We therefore used RNA interference (RNAi) to knock down the function of the RecA homolog *DMC1*, a meiosis-specific recombinase essential for the formation of crossovers. As RNAi is genetically dominant, it is easy to obtain progeny devoid of the RNAi cassette that would otherwise cause sterility phenotypes among reverse-breeding offspring. RNAi silencing is easy to implement in many crops, and a single cassette targeting a well-conserved meiotic gene can be used across multiple crop species. We used the *Brassica carinata* *DMC1* gene (91.1% identity to *A. thaliana* *DMC1*) to silence *A. thaliana* *DMC1* (Supplementary Fig. 1).

Among 50 RNAi-transformed plants, we observed a range of fertility phenotypes. These ranged from plants with siliques of the normal wild-type length to almost sterile plants (ten plants, 20% of the total) that harbored few or no seeds in their siliques (Supplementary Fig. 2) and produced irregularly sized pollen (Supplementary Fig. 3). Severely RNAi-transformed plants thus phenocopied the semi-sterile phenotype of *A. thaliana* *dmc1* mutants⁵. Transcriptional analyses

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of the RNAi cassette and the endogenous *DMC1* gene further confirmed the effective knockdown of the meiotic recombination pathway (Supplementary Fig. 4). For our experiments we selected those transformants that showed the most sterile phenotypes (that is, those carrying the shortest siliques).

Balanced gametes can be produced in the absence of chiasmata

By physically linking homologous chromosomes, chiasmata ensure their proper segregation during meiosis I. In the absence of crossovers (achiasmatic meiosis), non-recombinant chromosomes segregate randomly. This segregation is generally unbalanced, leading to aneuploidy in gametes and hence explaining the sterile phenotype of achiasmatic plants⁶. However, by chance balanced gametes are also produced at frequencies that depend on the plant's chromosome number³. *A. thaliana* ($n = 5$) theoretically produces 3.25% balanced gametes ($2^{-5} = 1/32$). Such numbers predict the ample production of viable pollen in the absence of crossovers, even for species with higher chromosome numbers.

We examined meiotic cell spreads in more than 100 diakinesis cells to confirm the absence of pachytene pairing and the presence of only univalents, which explained the absence of crossovers in our transformants (Fig. 1). Later meiotic stages also showed configurations that support the random segregation of chromosomes at meiosis I and meiosis II. We observed unbalanced tetrads and polyads predominantly at the end of meiosis. Analysis of 85 dyad-stage meiocytes using FISH showed occasional meiotic irregularities such as lagging chromosomes or chromatid segregation at meiosis I (Supplementary Fig. 5), and only two instances in which there was at least one balanced meiosis I product. This result seems close to the expected 3.25%. In addition, the low numbers of viable seeds suggest low-frequency production of balanced gametes, although exact numbers vary between independent T_1 plants, possibly because of variation in the expression of the RNAi transgene (Supplementary Table 1).

Conversion of balanced gametes into adult plants

To examine the effect of crossover silencing in gametes for reverse-breeding purposes, we constructed two near-isogenic *A. thaliana* hybrids, differing only in the presence of the *RNAi:DMC1* transgene. We crossed accessions Columbia (Col-0) and Landsberg *erecta* (*Ler*-0) to create a wild-type F_1 (WT F_1). We also crossed Col-0 carrying an *RNAi:DMC1* transgene with *Ler*, creating a reverse-breeding F_1 (RB F_1).

The second step in reverse breeding is to convert haploid gametes, carrying non-recombined chromosomes, into homozygous diploid adults. This can be achieved through different methods depending on the plant species⁷. In *A. thaliana*, haploid plants can be produced by centromere-mediated genome elimination⁸. We crossed both WT F_1 and RB F_1 pollen to the predominantly male sterile haploid inducer, creating wild-type and reverse-breeding haploids. We converted these haploids into fertile diploids (doubled haploids) by collecting the rare seeds following self-pollination of the haploids (see Online Methods).

We genotyped wild-type and reverse-breeding haploids and corresponding doubled haploids using evenly spaced SNP markers at approximately 4-Mb intervals (Supplementary Fig. 6 and Supplementary Table 2) to identify recombination events in parental meiosis (Fig. 2). For our calculations of recombination frequencies, we used the larger data set of recovered haploids. The vast majority of chromosomes in wild-type haploids were recombinant, with an average of 1.1 crossovers detected per chromosome in any given doubled haploid (Supplementary Table 3). A haploid typically shows half the number of the crossovers that occurred during meiosis, and it

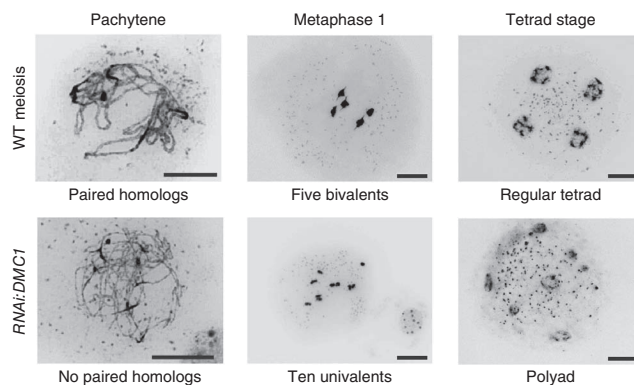


Figure 1 Meiosis in wild-type (WT; above) and *RNAi:DMC1* transformants (below). In wild-type meiosis, chromosomes pair at pachytene stage after which five bivalents are formed in metaphase 1. This results in tetrads showing four regular nuclei. In *RNAi:DMC1* transformants, tetrads are generally unbalanced, showing polyads, owing to unbalanced univalent segregation at metaphase 1. Suppression of *DMC1* also disrupts pairing of chromosomes at pachytene. Scale bars, 10 μ m.

will occasionally receive non-recombined chromatids from a bivalent. These non-recombinant chromosomes were usually restricted to a single chromosome, and no single plant was non-recombinant for all five chromosomes. As such, crossover recombination in our doubled-haploid population was comparable to that in previously published data^{9–12} in terms of genetic map length (crossover frequencies) as well as slight segregation distortions (see Online Methods).

Genetic analysis of 65 reverse-breeding haploids and their derived doubled haploids (that is, those plants resulting from achiasmatic meiosis) showed the complete absence of recombination, consistent with our cytological analyses (Fig. 1). Intact parental chromosomes segregated independently, leaving random assortment as the only process creating genetic variation among doubled haploids (Fig. 2c). Four additional reverse-breeding haploids did show crossovers (Fig. 2c), which might have resulted from accidental cross pollination or from incomplete silencing of *DMC1* by the RNAi construct (Supplementary Note).

The main objective of reverse breeding is to generate homozygous parental lines that can be mated to recreate a desired heterozygous genotype. In our set of non-recombinant reverse-breeding doubled haploids we identified 21 of the 32 (2^5) possible genotypes, including the original Col-0 parent (Fig. 2c). Notably, we could identify six sets of complementing parents—that is, genotypes that would reconstitute the initial hybrid when crossed. These complementing pairs are genetically distinct, and also differ from the original Col-0 and *Ler* parents. To complete reverse breeding, we made crosses between three pairs of selected reverse-breeding doubled haploid progeny to reconstitute the starting heterozygous parent (Fig. 2d). These crosses gave rise to perfectly heterozygous plants that were genetically identical to the achiasmatic Col/*Ler* hybrid parent (Supplementary Table 2).

Reverse breeding creates chromosome-substitution lines

Segregation of intact parental chromosomes also creates chromosome-substitution lines, in which a single chromosome is substituted by the corresponding homolog from a different line. Chromosome-substitution lines are valuable tools in many breeding applications, such as trait mapping, the study of epistatic interactions and targeted inbreeding¹³. Chromosome-substitution lines in *A. thaliana* have been generated through traditional crossing¹⁴; however, that method requires more generations of crossing and extensive genotyping to

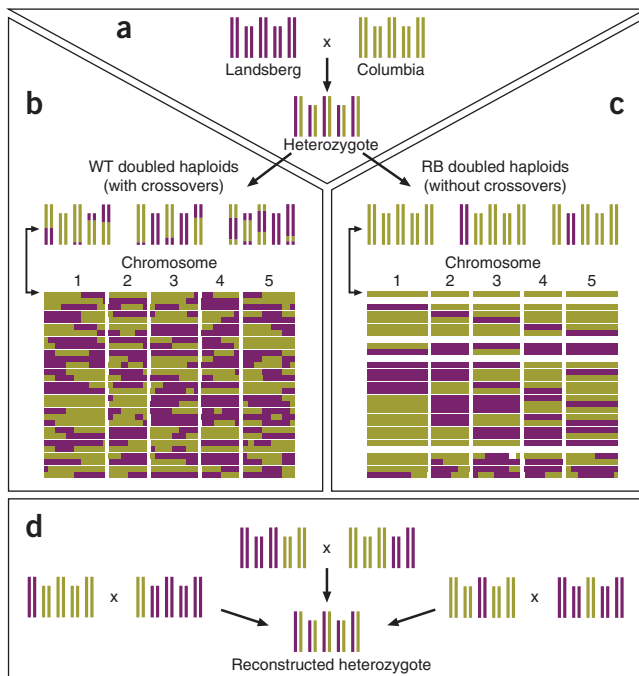


Figure 2 Reverse-breeding strategy and genotypes of wild-type (WT) and reverse-breeding (RB) doubled-haploid offspring. (a) Reverse breeding starts with a heterozygote in which meiotic recombination can be suppressed. (b) Genotype of 29 randomly selected wild-type doubled haploids. Three individuals are shown with 'classic' vertical chromosomes, but others as horizontal lines only. Each line represents chromosomes 1–5 for an individual plant. Note the presence of non-recombinant chromosomes. (c) We recovered 21 different genotypes in which no crossovers occurred from among 36 reverse-breeding doubled haploids. The first row represents the genotype of one of the recovered original parents; the next seven genotypes represent chromosome-substitution lines and the remainder are mosaics of Col and Ler chromosomes. The last four represent genotypes of haploid offspring that showed crossovers (Supplementary Note). (d) Three pairs of reverse-breeding doubled haploids were crossed to recreate the initial hybrid; they have the RNAi transgene.

identify a particular chromosome-substitution line. With reverse breeding, one can obtain all possible chromosome-substitution lines in a short period of time. In two generations, we obtained a complete set of *Ler* chromosome substitutions in the Col-0 background, as well as two substitutions of a Col-0 chromosome into a *Ler* background from our population of only 36 doubled-haploid plants (Fig. 2c).

DISCUSSION

Reverse breeding allows any desired heterozygote to be selected from a large population and be propagated indefinitely as F_1 . This is crucial, because it alleviates one of the limitations of traditional breeding in which hybrids are generated by controlled crossing using a low few founder lines. The genomes of uncharacterized heterozygous plants can now be fixed in complementing immortal lines without knowledge of their provenance. This ability to fix heterozygous genomes resembles apomixis, in which heterozygotes reproduce clonally through seeds², but is fundamentally different. If apomixis were to be engineered for crops¹⁵, the clonally propagated line would represent a dead end, as it could not be improved further by conventional hybrid breeding techniques. Because reverse breeding generates homozygous parental lines for the selected heterozygote, it allows the improvement of the heterozygote through improvement of the individual parental lines by traditional breeding methods such as backcrossing, mutagenesis and so on.

The technical feasibility of reverse breeding in *A. thaliana* suggests that it might be possible to apply this technology in crop improvement. Crucially, genes governing meiotic recombination are widely conserved¹⁶, and haploid generation methods are available for many crops⁷. Although the probability of recovering balanced gametes decreases with increasing chromosome numbers, many agronomically important crops have 12 chromosomes or fewer, and are within the reach of successful reverse breeding³. Notable examples include cucumber, onion, broccoli, cauliflower, sugar beet, maize, pea, sorghum, (water-) melon, tomato, pepper, rice, eggplant and so on. Reverse breeding might be difficult to adopt in crops with higher chromosome numbers (for example, soybean) as well as in polyploids such as canola, cotton, wheat and potato.

The use of a dominant RNAi transgene means that 50% of reverse-breeding offspring carry the transgene, rendering them semi-sterile. The use of inducible RNAi constructs could circumvent this caveat. Alternatively, one could select two independent transformants of the same heterozygote, such that the RNAi constructs are inserted on different chromosomes. Pairs of transgene-free parents can then be obtained. In our case, reverse-breeding doubled-haploid lines 17 and 61 do not contain the *RNAi:DMC1* transgene and would generate a transgene-free reconstructed heterozygote when crossed (Supplementary Table 2).

The expected low production of balanced gametes in crops with higher chromosome numbers is a possible bottleneck in reverse breeding. However, successful reverse breeding does not require the complete knockdown of crossovers, and the occurrence of a few crossovers might be beneficial, as non-recombinant chromatids are still present in bivalent pairs with a single crossover³. In rice ($n = 12$), for example, allowing three crossovers would already increase the chance of finding a balanced gamete from $2^{-12} = 1/4,096$ to $2^{-9} = 1/520$, at the expense of a lower recovery rate for true non-recombinant reverse-breeding offspring (1 out of 8 (2^{-3})). Our observation that different transformants showed varying degrees of sterility suggests that incomplete knockdown by RNAi could yield a desired level of crossover suppression. Segregation distortion in our population of haploid offspring (see Online Methods) was not extreme and was similar to that in previously described RIL populations¹¹. All chromosomes were still frequently transmitted to offspring.

Our ability to exert control over the complex outcomes of meiosis, together with the ever increasing need for new methods for crop improvement¹⁷, advocates for the rapid development of reverse breeding in crops. We therefore envision that new possibilities for the selection and improvement of favorable genotypes by reverse breeding may contribute to increasing future crop production.

URLs. *Arabidopsis* information resource, <http://www.arabidopsis.org/>; Joinmap, <http://www.kyazma.nl/>; KASPar SNP genotyping system, <http://www.kbioscience.co.uk/reagents/KASP.html>; MSQT, <http://msqt.weigelworld.org/>; pKANNIBAL vector, <http://www.pi.csiro.au/RNAi/vectors.htm>.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturegenetics/>.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

K.v.D., C.L.C.L., H.d.J. and R.D. conceived the research. E.W. performed cytology and crosses, C.B.d.S. constructed vectors and performed genotyping and N.S.N. performed cytology and FISH. E.W. analyzed data with the help of C.B.d.S., J.J.B.K., M.R. and S.W.L.C. E.W. wrote the manuscript and made the figures with substantial contributions by J.J.B.K., M.R., S.W.L.C. and H.d.J. All authors except N.S.N. were involved in planning and design of experiments, and read and improved the manuscript.

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- Chen, Z.J. Molecular mechanisms of polyploidy and hybrid vigor. *Trends Plant Sci.* **15**, 57–71 (2010).
- van Dijk, P. & van Damme, J. Apomixis technology and the paradox of sex. *Trends Plant Sci.* **5**, 81–84 (2000).
- Dirks, R. *et al.* Reverse breeding: A novel breeding approach based on engineered meiosis. *Plant Biotechnol. J.* **7**, 837–845 (2009).
- Ma, H. A molecular portrait of *Arabidopsis* meiosis. *Arabidopsis Book* **4**, e0095 (2006).
- Couteau, F. *et al.* Random chromosome segregation without meiotic arrest in both male and female meiocytes of a *dmc1* mutant of *Arabidopsis*. *Plant Cell* **11**, 1623–1634 (1999).
- Hartung, F. *et al.* The catalytically active tyrosine residues of both SPO11–1 and SPO11–2 are required for meiotic double-strand break induction in *Arabidopsis*. *Plant Cell* **19**, 3090–3099 (2007).
- Forster, B.P., Heberle-Bors, E., Kasha, K.J. & Touraev, A. The resurgence of haploids in higher plants. *Trends Plant Sci.* **12**, 368–375 (2007).
- Ravi, M. & Chan, S.W.L. Haploid plants produced by centromere-mediated genome elimination. *Nature* **464**, 615–618 (2010).
- Drouaud, J. *et al.* Sex-specific crossover distributions and variations in interference level along *Arabidopsis thaliana* chromosome 4. *PLoS Genet.* **3**, e106 (2007).
- Toyota, M., Matsuda, K., Kakutani, T., Terao Morita, M. & Tasaka, M. Developmental changes in crossover frequency in *Arabidopsis*. *Plant J.* **65**, 589–599 (2011).
- Alonso-Blanco, C. *et al.* Development of an AFLP based linkage map of Ler, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of a Ler/Cvi recombinant inbred line population. *Plant J.* **14**, 259–271 (1998).
- Hauge, B.M. *et al.* An integrated genetic/RFLP map of the *Arabidopsis thaliana* genome. *Plant J.* **3**, 745–754 (1993).
- Nadeau, J.H., Singer, J.B., Matin, A. & Lander, E.S. Analysing complex traits with chromosome substitution strains. *Nat. Genet.* **24**, 221–225 (2000).
- Koumproglou, R. *et al.* STAIRS: A new genetic resource for functional genomic studies of *Arabidopsis*. *Plant J.* **31**, 355–364 (2002).
- Marimuthu, M.P.A. *et al.* Synthetic clonal reproduction through seeds. *Science* **331**, 876 (2011).
- Mercier, R. & Grelon, M. Meiosis in plants: Ten years of gene discovery. *Cytogenet. Genome Res.* **120**, 281–290 (2008).
- Tester, M. & Langridge, P. Breeding technologies to increase crop production in a world. *Science* **327**, 818–822 (2010).

ONLINE METHODS

Plant material. *A. thaliana* plants were grown under standard conditions in a greenhouse. WT F₁ was obtained by Ler-0 (CS20) (female) × Col-0 (ABRC stock CS60000) (male). RB F₁ plants were made by crossing male Ler-0 to two semi-sterile RNAi:DMC1 transformants: T39 and T62 (in Col-0). For a third RB F₁ we used a male sterile (*ms1*^{-/-}) Ler (CS261) (for convenient crossing) pollinated with T62. WT F₁ and RB F₁ plants were crossed as the male to *cenh3-1 GFP-tailswap* females to generate haploids⁸. By using *GFP-tailswap* as a female, we aimed for the recovery of reverse-breeding gametes through microspores, as these greatly outnumber egg cells (and therefore increases the probability of recovering balanced gametes).

Our crosses of both WT F₁ and RB F₁ to *GFP-tailswap* females yielded populations of diploid, haploid and aneuploid offspring, the last group resulting from incomplete genome elimination. Haploids were identified by their homozygous genotype and vegetative phenotype (semi-sterile flowers, smaller rosette size and narrow leaves compared to diploids)⁸. In the offspring of the RB F₁ plants we also recovered semi-sterile diploids as a result of the RNAi construct. Aneuploids were discarded based on their aberrant growth phenotypes. The WT F₁ to *GFP-tailswap* crosses yielded 73% of haploids, whereas the RB F₁ to *GFP-tailswap* cross resulted in 42% of haploids. Recovery rates for aneuploids were low (~3%) in both populations.

We obtained no seeds for 56% and 55% of doubled haploids from the wild-type and reverse-breeding haploid populations, respectively. This was presumably in part due to growth conditions (on soil). We have found later that, when haploids are grown to larger sizes (on rock wool), haploid plants produce more seeds (data not shown).

Plant transformation. A 293-bp sequence of the *B. carinata* cDNA (Supplementary Fig. 1) was PCR amplified to clone the *DMC1* coding sequence, of which both sense and antisense orientation were cloned into a pKANNIBAL hairpin RNAi vector (CSIRO). The vector was subsequently cloned in an pART27 binary vector¹⁸ and transformed into Col-0 using floral dip¹⁹.

Quantitative RT-PCR. The nucleic acid was extracted from 10 mg of unopened flower buds pooled from several inflorescences. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Real-Time PCR was done using iQ SYBR Green Supermix (Bio-Rad) on the CFX96 Real-Time PCR Detection System (Bio-Rad). The ΔC_t was calculated using *SAND* and *UBC* as endogenous controls²⁰. We calculated relative quantification values (RQ) by the $2^{-\Delta C_t}$ method (RQ = $2^{-\Delta C_t}$); results represent the average of five biological replicates. Oligonucleotides used for real-time PCR are given in Supplementary Table 4). For more details about the RNAi construct and the coordinates of *AtDMC1* oligos used for real-time PCR, please refer Supplementary Figure 1.

Microscopy and fluorescence in situ hybridization. Pollen preparations were stained with lactophenol acid fuchsin. Meiotic preparations and FISH followed standard protocols^{21–23}. The probes used and their position on *A. thaliana* chromosomes are shown in Supplementary Figure 5. Slides were examined under a Zeiss Axioplan 2 imaging photomicroscope equipped with epifluorescence illumination and filters.

Genetic analysis. SNPs were selected using MSQT²⁴ and matched to the Columbia reference genome using the *Arabidopsis* information resource. Genotyping was done using the KASPar SNP genotyping system. Joinmap version 4.1 (ref. 25) was used for calculation of recombination frequencies, and χ^2 tests were used for segregation distortions. Recombination frequencies were estimated using the regression mapping algorithm with 'independence LOD' as grouping parameter and the Kosambi mapping function to convert recombination frequencies to map distances.

Marker segregation in wild-type and reverse-breeding haploids. During marker analysis, we noticed clear segregation distortions in both wild-type and reverse-breeding haploids. Among wild-type haploids, Col alleles are overrepresented at the lower end of chromosome 1, the top and bottom of chromosome 2, the middle of chromosome 4 and at the top of chromosome 5 (Supplementary Fig. 7). Segregation distortions have previously been studied in a Col/Ler recombinant inbred line (RIL) population^{26,27}. The overrepresentation of Col alleles at the lower end of chromosome 1 in that population is concurrent with our observations, possibly having similar causes. Notably, the Col alleles on chromosomes 2 and 5 were overrepresented in our haploid population, in contrast to the overrepresentation of the *Ler* alleles in the RIL population. A previous report described a genetic incompatibility leading to segregation distortions at the lower end of chromosome 1 and the top of chromosome 5 in *A. thaliana*²⁸. Nonetheless, this is not expected to be causing the segregation distortions found in these populations, as Col and *Ler* share the same alleles for these loci.

Because, in reverse breeding, linkage is absolute for all loci on a chromosome, any distorting locus would affect transmittance of the whole chromosome. The overrepresentation of Col chromosomes 1, 4 and 5 in the reverse-breeding haploids could be the direct effect of such distorting loci. For these chromosomes it seems plausible that this is caused by the same loci causing the preferential transmittance of Col alleles to the wild-type haploids. Notably, the overrepresentation of Col alleles at the top of chromosome 2 is not matched by an overrepresentation of that chromosome in reverse-breeding haploids. This might be caused by a 'balancing' mechanism, as at the lower end of chromosome 2 there seems to be a locus of which the Col allele is transmitted less frequently than its *Ler* counterpart.

We hypothesize that the increased genetic linkage imposed upon the alleles on one chromosome in reverse breeding may strongly affect the transmittance of alleles. When linkage becomes absolute, distorting alleles may act either directionally or in a balancing manner, depending on the sign of their effect.

The differences in segregation distortions between the Col/Ler RILs and the haploids may also lie in the methods used for the construction of these populations. Our haploids were produced using only male meiosis, whereas, in the RILs, gametes produced by female meiosis also contribute to observed effects. Second, the haploids underwent a process of genome elimination, and it is possible that there are alleles that favor either their genesis or the survival of haploid embryo.

18. Gleave, A.P. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20**, 1203–1207 (1992).
19. Clough, S.J. & Bent, A.F. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743 (1998).
20. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. & Scheible, W.-R. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* **139**, 5–17 (2005).
21. Ross, K.J., Fransz, P. & Jones, G.H. A light microscopic atlas of meiosis in *Arabidopsis thaliana*. *Chromosome Res.* **4**, 507–516 (1996).
22. Ross, K.J. *et al.* Cytological characterization of four meiotic mutants of *Arabidopsis* isolated from T-DNA-transformed lines. *Chromosome Res.* **5**, 551–559 (1997).
23. Tang, X. *et al.* Cross-species bacterial artificial chromosome-fluorescence in situ hybridization painting of the tomato and potato chromosome 6 reveals undescribed chromosomal rearrangements. *Genetics* **180**, 1319–1328 (2008).
24. Warthmann, N., Fitz, J. & Weigel, D. MSQT for choosing SNP assays from multiple DNA alignments. *Bioinformatics* **23**, 2784–2787 (2007).
25. Stam, P. Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. *Plant J.* **3**, 739–744 (1993).
26. Lister, C. & Dean, C. Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. *Plant J.* **4**, 745–750 (1993).
27. Singer, T. *et al.* A high-resolution map of *Arabidopsis* recombinant inbred lines by whole-genome exon array hybridization. *PLoS Genet.* **2**, e144 (2006).
28. Bikard, D. *et al.* Divergent evolution of duplicate genes leads to genetic incompatibilities within *A. thaliana*. *Science* **323**, 623–626 (2009).