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Partial cytological diploidization of neoautotetraploid meiosis by induced cross-over rate reduction

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Polyploids, which arise from whole-genome duplication events, have contributed to genome evolution throughout eukaryotes. Among plants, novel features of neopolyploids include traits that can be evolutionarily or agriculturally beneficial, such as increased abiotic stress tolerance. Thus, in addition to being interesting from an evolutionary perspective, genome duplication is also increasingly recognized as a promising crop improvement tool. However, newly formed (neo)polyploids commonly suffer from fertility problems, which have been attributed to abnormal associations among the multiple homologous chromosome copies during meiosis (multivalents). Here, we test the long-standing hypothesis that reducing meiotic cross-over number may be sufficient to limit multivalent formation, favoring diploid-like bivalent associations (cytological diploidization). To do so, we developed *Arabidopsis thaliana* lines with low cross-over rates by combining mutations for *HEI10* and *TAF4b*. Double mutants showed a reduction of ~33% in cross-over numbers in diploids without compromising meiotic stability. Neopolyploids derived from the double mutant show a cross-over rate reduction of about 40% relative to wild-type neotetraploids, and groups of four homologs indeed formed fewer multivalents and more bivalents. However, we also show that the reduction in multivalents comes with the cost of a slightly increased frequency of univalents and that it does not rescue neopolyploid fertility. Thus, while our results do show that reducing cross-over rates can reduce multivalent frequency in neopolyploids, they also emphasize that there are additional factors affecting both meiotic stability and neopolyploid fertility that will need to be considered in solving the neopolyploid fertility challenge.

polyploidy | recombination rate | multivalent | autotetraploid

Significance

Polyploidy, or whole-genome duplication, has occurred throughout eukaryotes, especially plants. Polyploids often exhibit advantages such as larger fruits, seeds, or leaves, and resilience to climate-stressors like drought, likely explaining why polyploids are overrepresented especially among plants. Unfortunately, the use of newly formed neopolyploids in crop improvement is often hampered by their low fertility—a trait often correlated with meiotic defects. Based on prior understanding of how natural polyploids evolved solutions to meiotic challenges, here we test the hypothesis that reducing meiotic recombination rates could be a useful engineered solution for this problem. We show that indeed a cross-over rate reduction does mitigate some meiotic defects, but also that this is not the whole problem.

Polyploidy, or whole-genome duplication (WGD), is thought to have contributed substantially to the evolution of genomic and organismal complexity across eukaryotes, especially in plants (1–3). The prevalence of polyploids among crop species is attributed to the fact that they commonly have novel phenotypes, including larger organ size and strong resilience to a range of climate-relevant abiotic stresses (4). Thus, within-species genome duplication (leading to autopolyploidy) has been flagged as a potentially important crop improvement tool (5, 6). Yet, successful new neoautopolyploid agricultural varieties remain rare (6, 7). An important obstacle to the wider use of induced polyploidy in agriculture mirrors that facing natural neopolyploids, namely the poor fertility and genome instability of neopolyploids (8), which are thought to arise largely as a consequence of meiotic errors (Fig. 1) (8–15). To understand polyploid evolution and enable the wider application of polyploidy for crop improvement, testing hypotheses about how neopolyploid meiosis can be stabilized is important (7, 16).

A common challenge in neopolyploid meiosis is the formation of multivalent associations among the multiple copies of each homologous chromosome (Fig. 1). The correlation between multivalent formation and reduced fertility (10, 17, 18), and the observation that evolved polyploids form fewer multivalents than neopolyploids (15), hint that multivalents are a problem that must be solved after WGD. To understand why neopolyploids form multivalents and why these are problematic, it is helpful to first consider what happens in diploid meiosis: For proper, error-free meiosis in diploids, the two homologous copies of every chromosome (homologs) associate in pairs through the polymerization of the synaptonemal complex (forming synaptic bivalents bivalents) during meiotic prophase I. During this time, homologs also undergo reciprocal exchanges of genetic material (cross-overs). Cross-over events not only drive genetic exchange, they are also required to help hold the homologous chromosomes together as bivalents into metaphase I (Fig. 1), which in turn is essential for the precise segregation of homologous chromosomes in meiosis I (19). Each homolog pair must establish at least one cross-over (cross-over assurance) (20, 21) to prevent missegregation and aneuploidy. In polyploids, cross-overs are a double-edged sword; they are essential for chromosome segregation—as they are in

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diploids—but may become problematic when at metaphase I they link more than the usual two chromosomes into multivalent associations (which in a tetraploid include quadrivalents and trivalents) and some unpartnered univalents, which pose challenges during chromosome segregation (18). Multivalent occurrence is correlated with reduced fertility. The most dangerous configuration four chromosomes can take on is apparently the "trivalent plus univalent" configuration as it leaves one unpartnered chromosome, which is prone to loss or missegregation (22–24). Most natural (established) polyploids have evolved solutions to prevent multivalent associations (18, 25) and thus form mostly or only bivalents in metaphase I (Fig. 1). This adaptive process of restoring diploid-like bivalent formation and regular chromosome segregation is referred to as "cytological diploidization"(26) and is associated with recovery of fertility in many species (8, 10, 11, 24, 26).

The evolved meiotic modifications leading to cytological diploidization differ between two major types of polyploid, autopolyploids, and allopolyploids. Allopolyploids form from interspecies or interstrain hybridization coupled with genome duplication (2, 27). Allopolyploids stabilize meiosis primarily by ensuring preferential recombination among homologs from the same genomic origin, i.e., the same subgenome, and the genetic basis of this type of stabilization is known for some cases (reviewed in refs. 28 and 29). Autopolyploids, in contrast, originate from within-species WGD and thus have multiple equally homologous copies of each chromosome (2, 27). Autopolyploids are common in many natural systems, and enticing for generating crop strains as they do not have the added complication of hybridity, but how their initial fertility problems can be solved is mostly mysterious. Given that more cross-overs intuitively increase the likelihood of a chromosome associating with multiple partners (Fig. 1), it has been hypothesized that reducing cross-over frequency could suffice to reduce multivalent formation rates in neoautopolyploids, and suggestive correlations have indeed been observed in several natural polyploids [(17, 23, 30, 31); for review ref. 18)]. However, it may not be as simple as it seems: Reducing the rate of cross-overs might indeed reduce quadrivalent frequencies, but could at the same time increase the frequency of meiotic instability if it results in a higher frequency of univalents. Indeed, other mechanisms such as altered cross-over positioning or increased cross-over interference have been suggested to play a role as well (11, 24, 32–34). Therefore, although cytological diploidization is often accompanied by cross-over rate reduction, whether this alone can suffice to stabilize meiosis has not been explicitly tested.

To test the hypothesis that cross-over rate reduction can lead to an increase in bivalents and a decrease in multivalents, we need to be able to manipulate cross-over rates without disrupting meiosis. Multiple genes are known that can in theory be used to manipulate cross-over rates, but many cause severe disruptions of meiosis (35, 36). In eukaryotes, there are two types of cross-overs, class I and class II, of which the latter are less frequent; in *Arabidopsis thaliana*, about 85% of all cross-overs are class I (21, 37). Class I (but not Class II) cross-overs are subject to cross-over interference, which prevents their close spacing and also plays a role in cross-over assurance (20, 21, 38, 39). For class I cross-over formation, several proteins are required, but one, HEI10, is particularly interesting here because it promotes class I cross-over formation in a dosage-dependent manner and variations in its expression levels quantitatively affect cross-over rates (40–42). Current models suggest that HEI10 may also be important for cross-over interference (39, 43, 44). Another gene known to quantitatively diminish cross-over rates is *TAF4b*, for which a naturally occurring mutant exists in *A. thaliana* that has reduced cross-over rates (45). *TAF4b*

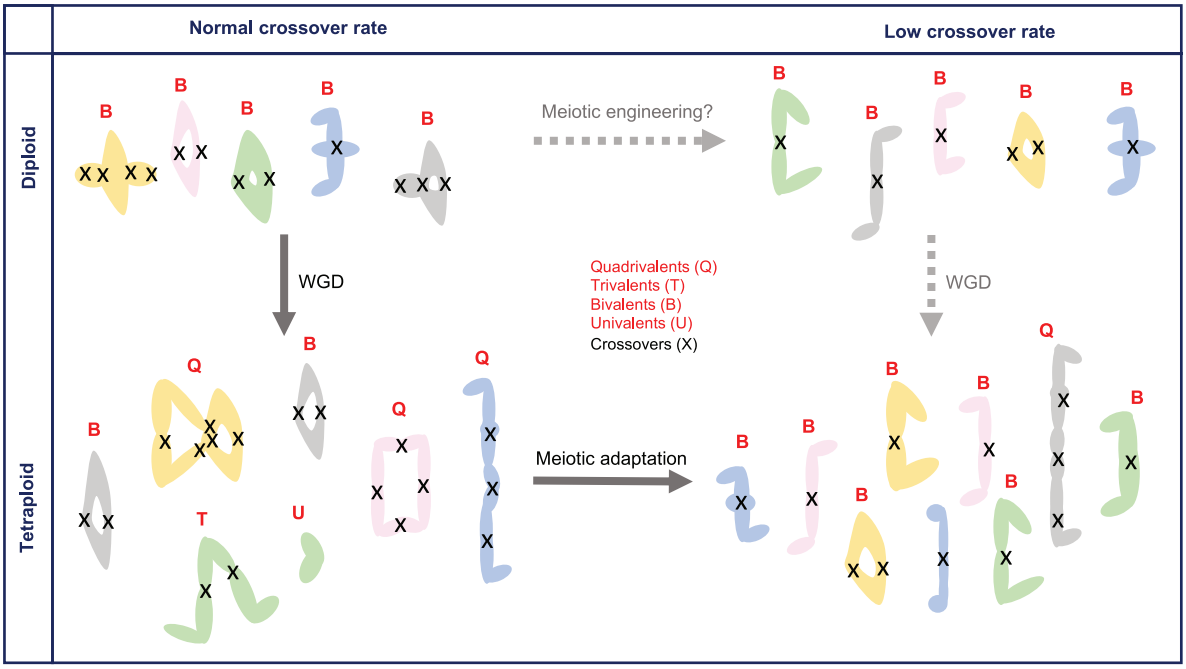

Fig. 1. Cytological diploidization by evolved and engineered cross-over rate reduction. This model represents how, in an imaginary species with a diploid karyotype formed by 5 pairs of chromosomes (2× = 2n = 10, each color represents one homolog pair) and with a given number of meiotic cross-overs (x), after WGD, can achieve cytological diploidization. Solid arrows represent the observed evolutionary pathway—based on meiotic adaptation—whereas dashed arrows illustrate the engineered solution—by inducing cross-over rate reduction—that we test in this study. In diploids with normal cross-over rate, bivalents (B) are readily formed. By contrast, after WGD, the resulting neotetraploid (4× = 2n = 20, each color represents a group of four homologs) bivalent formation is compromised. Instead, due to a doubled number of chromosomes and cross-overs, meiotic irregularities such as quadrivalents (Q), trivalents (T) and univalents (U) are commonly formed in neotetraploids. As typically observed, after numerous generations of meiotic adaptation, diploid-like bivalent formation can be restored (achieving cytological diploidization), commonly accompanied by a cross-over rate reduction.

encodes a reproduction-specific variant of the core transcription factor TAF4, a subunit of the RNA Polymerase II general transcription factor TFIID. In plants and animals alike, *TAF4b* regulates core meiotic genes and likely affects recombination by altering the expression of recombination genes (45–47).

Here, we test the hypothesis that reducing cross-over frequency can promote cytological diploidization in neopolyploids by increasing the frequency of metaphase I bivalents. We do this by creating *A. thaliana* lines with reduced class I cross-over numbers (but retaining cross-over assurance) by combining *TAF4b* and *HEI10* mutations and then use these lines to generate neotetraploids. We then ask if the double-mutant "low cross-over" (LCO) lines have reduced multivalent frequencies compared to neotetraploids created from wild type. We show that, as hypothesized, this engineered cross-over reduction indeed decreases neotetraploid multivalent frequency, thus driving partial cytological diploidization. But our results also show that this multivalent reduction comes with a cost of slightly elevated univalent formation rates, and that the reduction in multivalents does not rescue fertility. Thus, in both engineered and natural solutions to the fertility challenges of polyploids, there is more to fix than just multivalents. Based on our current and previous results, we hypothesize that the solutions to polyploid meiosis likely have at least two components: cross-over rate reduction, and regulation of relative cross-over positioning on four chromosome groups, possibly through increased cross-over interference.

Results

LCO Diploids Show Strong Cross-Over Reduction with Robust Cross-Over Assurance. To test whether reducing cross-over rate is sufficient to prevent multivalent formation in neotetraploids, we first needed to develop a reliable method to reduce cross-over number in diploids without affecting meiotic stability (i.e., without the loss of cross-over assurance). To attempt this, we used *A. thaliana* mutant genotypes in the Col-0 background that have been reported to show mild cross-over reductions of ~20% each without compromising obligate cross-over formation: heterozygotes for the *hei10-2* mutation [*hei10-2*/+, (42)] and homozygotes for the *taf4b-2* mutant allele (45). Since they likely act in separate pathways, we hypothesized that combining these two mutations might lead to an even lower cross-over rate.

For a precise measurement of class I cross-over formation rate, we used HEI10 immunostaining in late prophase I male meiocytes (diakinesis and diplotene, Fig. 2 *A* and *B* and Dataset S1). In Col-0 WT, we observed 10.0 HEI10 foci per cell (±1.4, n = 44 cells in 1 plant) on average [in agreement with previous studies (21, 39, 40)]. As expected from previous literature (42,

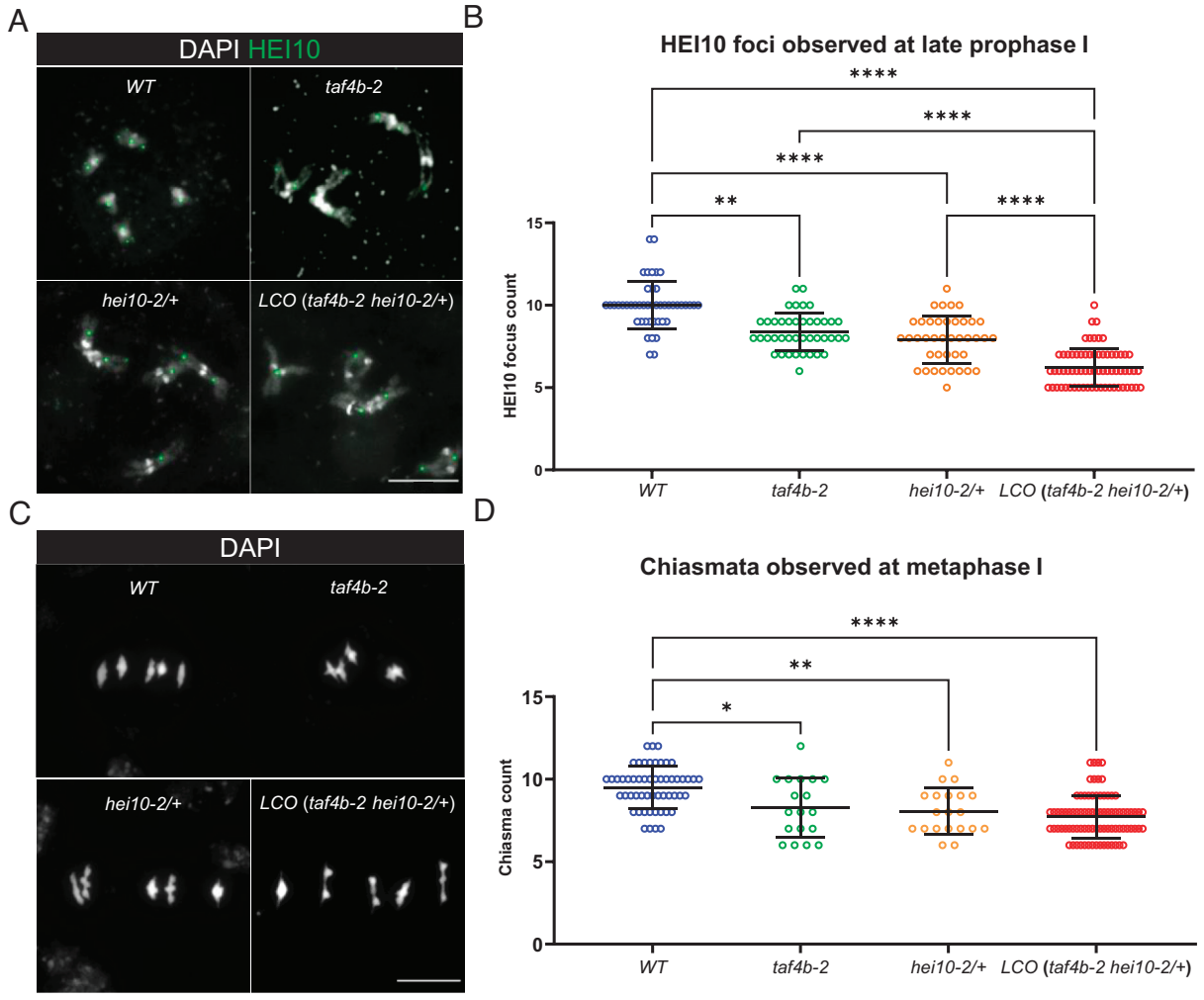

Fig. 2. Cross-over reduction in LCO genotype. (*A*) Examples of HEI10 immunolocalization on diakinesis chromosomes. (Scale bar, 10 μm.) (*B*) Differences observed for HEI10 foci number in different genotypes with a horizontal bar showing mean, and bars indicating SD. (*C*) Examples of metaphase I spreads stained with DAPI. (Scale bar, 10 μm.) (*D*) Differences in chiasma number between genotypes with a horizontal bar showing mean, and bars indicating SD. Statistical significance is indicated in panels *B* and *D* based on Kruskal-Wallis test, with ****P-value < 0.0001, **P-value < 0.01, *P-value < 0.05.

45), single mutants *taf4b-2* or *hei10-2*/+ displayed mild but significant reductions in HEI10 focus counts to 8.4 (±1.2, n = 41 cells in 2 plants) and 7.9 (±1.4, n = 40 cells in 2 plants) per cell, respectively (Kruskal–Wallis test, $P < 10^{-4}$). In the double mutant, we observed an even stronger reduction, to 6.2 foci (±1.1, n = 68, cells in 2 plants) per cell, which is significantly lower than the other genotypes analyzed (Fig. 2*B*, Kruskal–Wallis test, $P < 10^{-4}$). Considering that on average an additional 1.5 Class II (HEI10-independent) cross-overs per male meiocyte are expected in *A. thaliana*, these results suggest that the double mutant has an ~33% reduction in total cross-over number relative to wild type. Importantly, we observed neither univalents at metaphase I, nor any reduction in fertility when assayed by pollen viability or seed production (*SI Appendix*, Fig. S1).

HEI10 counts, while accurate, only assay class I cross-overs, so we also analyzed meiotic spreads at metaphase I for all the genotypes, where both class I and class II cross-overs can be quantified by counting chiasmata (48). Despite the lower reliability of this approach (because bivalent shapes can be ambiguous in some cases), we observed the same trend as above, with Col-0 WT showing the highest chiasma count per cell (9.5 ± 1.3, n = 54 cells in 6 plants, Fig. 2*C* and Dataset S1) and the double mutant the lowest (7.7 ± 1.3 chiasmata per cell, n = 84 cells in 10 plants; Kruskal–Wallis test, $P < 10^{-5}$). Though the double mutant had fewer chiasmata than either *taf4b-2* (8.3 ± 1.8 chiasmata, n = 18 cells in 2 plants) or *hei10-2*/+ (8.1 ± 1.4 chiasmata per cell, n = 20 cells in 3 plants) the difference was not significant (Kruskal–Wallis test, Dataset S1). We named the double-mutant line LCO and used this in our further analyses.

LCO Neoautotetraploids Display Partial Cytological Diploidization. To study the effect of reducing cross-overs in progenitor diploids on multivalent formation in neotetraploids (4×) derived from them, we used colchicine to induce WGD in Col-0 WT and LCO genotypes. We then compared male metaphase I in WT and LCO neotetraploids using cytology. In order to tell the different chromosomes apart, we used fluorescence in situ hybridization (FISH) with probes corresponding to 45S and 5S rDNA (Fig. 3*A*), which allows identification of each of the five *A. thaliana* chromosomes and their respective arms, except Chromosome 1, where arms are not distinguishable (49). First, we counted chiasmata to verify whether reduced cross-over frequency in LCO relative to wild type was maintained in neotetraploids (50, 51). In neotetraploid WT we observed 19.5 ± 2.6 chiasmata per cell (n = 73 cells in 5 plants) in agreement with previous studies (50, 52), and in LCO we observed 15.5 ± 2.1 chiasmata per cell (n = 64 cells in 3 plants), a statistically significant reduction of 21% (Fig. 3*B*, Mann–Whitney *U* test, $P < 10^{-5}$). This difference, which is echoed for each of the 5 chromosomes individually (*SI Appendix*, Fig. S2 and Dataset S2), indicates that the cross-over reduction in diploid LCO is largely preserved in neotetraploids. For a more precise quantification of the change in class I cross-over rate, we used HEI10 immunostaining of late prophase I cells (Fig. 3*C*). We observed that in the tetraploid background, WT and LCO meiocytes nearly doubled their total diploid chiasma counts, meaning the per chromosome rate remained about the same in neopolyploids. Wild-type neotetraploids had 22.3 ± 0.9 HEI10 foci (n = 25 cells in 1 plant), while LCO neotetraploids had 11.9 ± 0.4 HEI10 foci per cell (n = 20 cells in 2 plants for WT and LCO; Fig. 3*D*). This difference was highly significant (Mann–Whitney *U* test, $P < 10^{-5}$). Assuming the per cell class II cross-over rate also doubles upon WGD, our results suggest that LCO neotetraploids show a ~41% total cross-over reduction relative to WT, which is slightly greater than the 33% observed in diploids (one sample *t* test, $P < 10^{-4}$).

Next, we tested whether the reduced cross-over rate in neotetraploid LCO induces a change in the frequency of chromosome associations observed in metaphase I relative to WT (i.e., whether they form quadrivalents, trivalents, bivalents, and univalents). In contrast to diploids, where chromosomes exclusively form bivalent associations during metaphase I, neotetraploids, regardless of their genotype, show a mixture of all possible associations. We found that the frequency of these different associations diverged between WT and LCO neotetraploids (Fig. 3*E*). In WT neotetraploids, 44% of four-homolog groups were associated as quadrivalents (224 quadrivalents out of 507 associations scored in 73 cells in 5 plants), while in LCO only 32% were quadrivalents (158 out of 487 associations in 64 cells in 3 plants, Fisher's exact test adjusted $P = 0.0008$ using Holm–Bonferroni correction). LCO neotetraploids accordingly had a modest but statistically significantly increased frequency of bivalents relative to wild type (61.4%; 299 bivalents out of 487 scored associations in LCO vs. 53.3%; 271 bivalents out of 507 scored associations in WT, Fisher's exact test $P = 0.025$ adjusted with Holm–Bonferroni correction; Fig. 3*F*). Furthermore, we analyzed how often groups of four homologous chromosomes (four-homolog groups) formed two bivalents (2B configuration; diploid-like behavior). WT neotetraploids showed 1.8 ± 1.2 2B configurations per cell (n = 73 cells in 5 plants) while LCO neotetraploids had significantly more, 2.3 ± 1.3 2B configurations per cell (n = 64 cells in 3 plants; Fig. 3*D*, Mann–Whitney *U*, $P = 0.0372$). In addition, we studied the distribution of the frequencies of meiocytes with 0 to 5 four-homolog groups in the 2B configuration and found strongly significant differences between WT (n = 365 four-homolog groups in 73 cells in 5 plants) and LCO (n = 320 four-homolog groups in 73 cells in 5 plants) neotetraploids (Fig. 3*G*; n = goodness of fit chi-square, $P < 10^{-5}$). Overall, the proportion of four-homolog groups with the 2B was ~9% higher in the LCO neotetraploids (46.0%; 147 2B configurations out of 320 four-homolog groups) relative to WT neotetraploids (37.0%; 135 2B configurations out of 365 four-homolog groups). Overall, these analyses suggested that LCO neotetraploids, display partial cytological diploidization, compared to WT, as the probability of forming "cytologically diploidized" (2B) configurations increased. We found that the 2B formation rate varied among chromosomes, with the improvement seen in LCO lines being the strongest for the smaller Chromosomes 2, 3, and 4 (22.6, 26.2, and 22.3 Mb respectively (53), for which 2B formation rate increased by ~9%, 23%, and 11%, respectively, in LCO neotetraploids relative to WT (30, 35 and 33 2B configurations out of 64 four homolog groups scored for Chromosome 2,3 and 4, respectively, for LCO and 28, 23, and 30 2B configurations in 73 four-homolog groups scored for Chromosome 2, 3, and 4, respectively, for WT. The larger Chromosomes, 1 and 5 [32.7 and 30.1 Mb, respectively, (54)] showed only a negligible increase in 2B formation rate in the LCO neotetraploids (1.9% and 0.7%, respectively).

Though LCO neotetraploids did show a significant decrease in multivalent frequency, we did see that univalents were ~3% more frequent in the LCO neotetraploids (1.38%; 7 univalents out of 507 scored associations of in WT vs. 4.11%; 20 univalents out of 487 scored associations in LCO neotetraploids; Fisher's exact test $P = 0.031$, adjusted with Holm–Bonferroni correction), though we did not observe this in diploids. We also observed a slight, but statistically nonsignificant increase in the proportion of associations scored as trivalents in LCO neotetraploids (0.99%; in WT vs. 2.05% in LCO neotetraploids; Fisher's exact test $P = 0.199$ adjusted with Holm–Bonferroni correction). These observations suggest that cross-over rate reduction in LCO increased the frequency of bivalents, but also univalents, at the expense of quadrivalents.

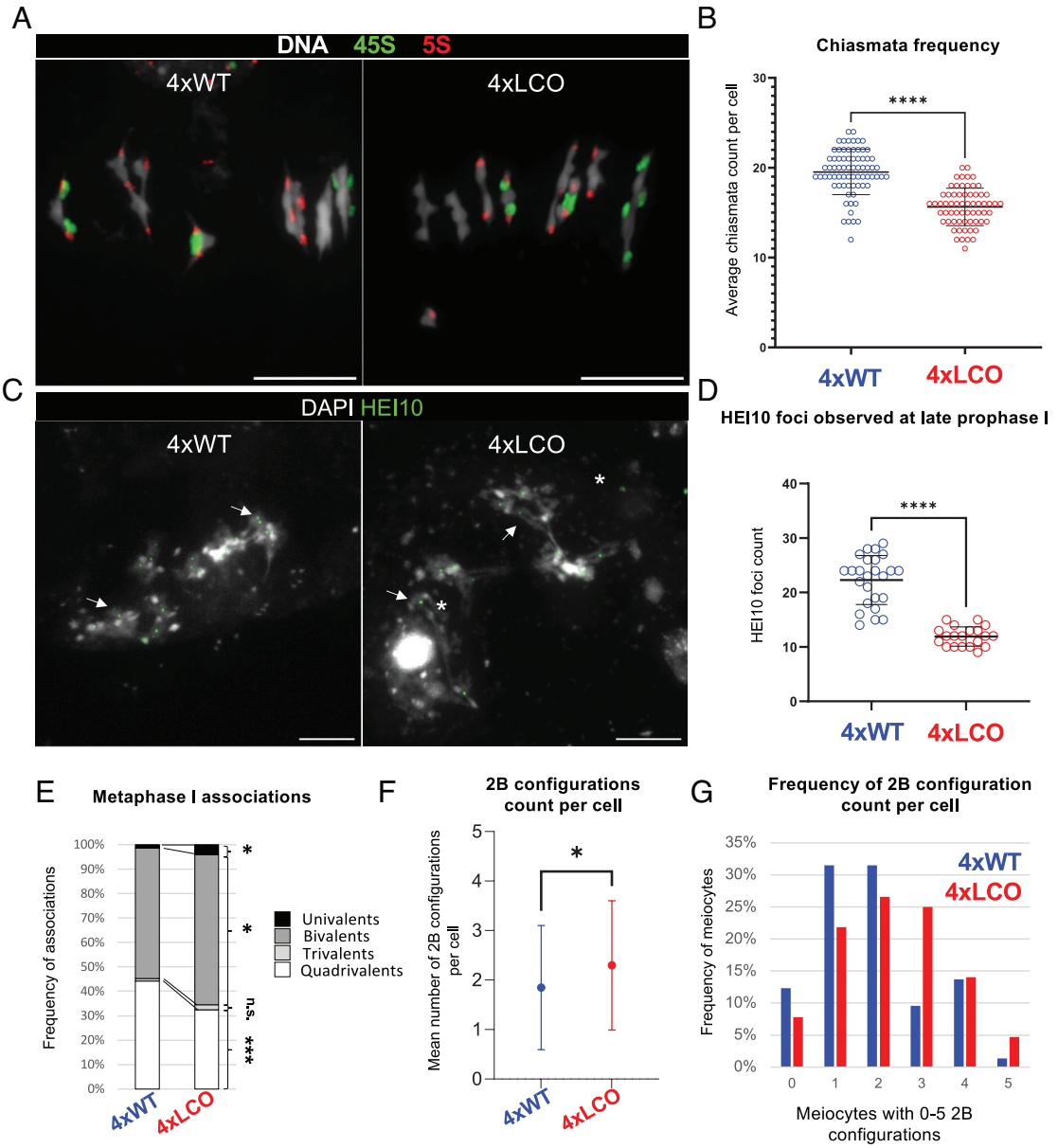

Fig. 3. Meiotic diploidization of LCO neotetraploids. (*A*) Examples of metaphase I spreads analyzed by FISH. (Scale Bar, 10 μm.) (*B*) Plot showing the results of chiasmata counting in WT and LCO neotetraploids (4×), with a horizontal bar showing mean, and bars indicating SD. (*C*) Examples of HEI10 immunolocalization on late prophase I chromosomes. Arrowheads point at examples of signals considered as true HEI10 foci whereas stars highlight examples of background signals that were not counted as HEI10 foci. (Scale bar, 10 μm.) (*D*) Plot showing differences in HEI10 counts between WT and LCO neotetraploids (4×). (*E*) Distribution of the frequencies of quadrivalent, trivalent, bivalent and univalent associations observed in neopolyploids. (*F*) Plot showing differences in diploid like behavior [i.e., mean number of 2B four-homolog groups (2B configurations) per cell]. (*G*) Histogram showing the frequency of meiocytes with 0 to 5 four-homolog groups forming two bivalents (2B configurations). Statistical significance is indicated in panels *B*–*E* based on *t* test, Fisher's exact test, Mann–Whitney *U*, and independence chi-square test, respectively, with ****P-value < 0.0001, ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05.

We checked if the partial cytological diploidization we observed improved neopolyploid fertility, but we did not observe significant differences in seed set or pollen viability between WT and LCO neotetraploids (*SI Appendix*, Fig. S1 and Dataset S3). This finding suggests either that multivalents are not the only cause of neopolyploid sterility (the hypothesis we favor) and/or that the ~40% cross-over reduction we achieved here was not enough to increase fertility even though it was enough to reduce multivalent formation rates.

Reduced Cross-Over Frequency Suffices to Explain Partial Cytological Diploidization in LCO Neotetraploids. In principle, a reduction in cross-over frequency can directly result in increased bivalent frequency (Fig. 4*A*). However, in the closely related *Arabidopsis arenosa*, we previously found that increased efficiency of cross-over interference is also an important feature of increased meiotic stability in the natural autotetraploid lineage that helps prevent trivalent + univalent (1T1U) configurations in four-homolog groups with two cross-overs (Fig. 4*A*, solution 2) (15). HEI10 has been previously implicated in cross-over interference, and reducing HEI10 dosage should yield wider spaced foci that can indicate stronger interference (39, 42, 44), so it could in principle also produce such an adaptation. Thus, we wished to explore two nonexclusive possibilities for the increase in bivalent frequency in LCO neotetraploids: 1) We reasoned that LCO tetraploids could have reduced multivalent and increased bivalent frequencies simply

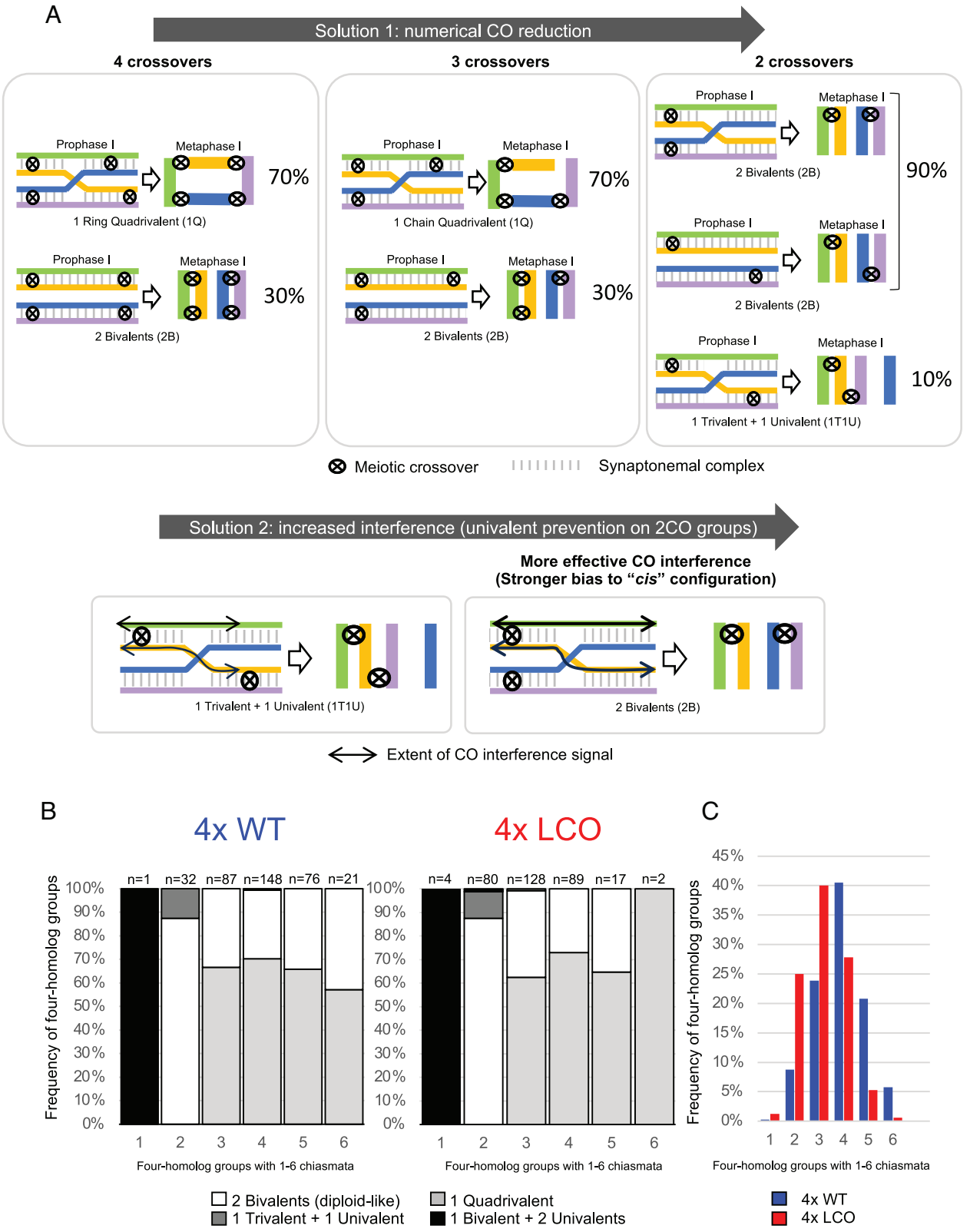

Fig. 4. Potential drivers of cytological diploidization in an autopolyploid. (*A*) Two hypotheses for adaptations leading to cytological diploidization. The four copies of each chromosome forming four-homolog groups are represented by four lines of four different colors and the switch points in the middle are synaptic partner switches, which occur in many but not all four chromosome groups. The *Top* panel "Adaptation 1" shows how a reduction in cross-over number per meiosis leads to a higher frequency of four chromosome groups that yield two bivalents. The frequencies of the different outcomes (e.g. Q vs. 2B) are based on the data shown in panel *B*. The *Rightmost* panel shows why the increase in two-cross-over chromosome groups also yields a low rate of univalent formation via the 1T1U configuration appearing more frequently than when more COs are present. The *Lower* panel shows "Adaptation 2" which, as described in the text, was based on observations in *A. arenosa* but was tested for here. In this scenario, the "cost" of two cross-over groups having a higher chance of producing the 1T1U configuration is reduced by an increased effectiveness of cross-over interference, such that cross-overs forming on opposite sides of an SPS site (a pre-requisite for 1T1U) becomes even rarer than it naturally is, leading to an even stronger bias toward the 2B configuration. (*B*) The distribution of configurations of four-homolog groups in WT and LCO neotetraploids (4×). (*C*) Frequency histogram of four-homolog groups with 1 to 6 chiasmata in both WT and LCO neotetraploids.

because of the increased likelihood of four-chromosome groups receiving only two cross-overs. This would come with a slight increase in univalents due to the higher likelihood of producing 1T1U configurations (solution 1 in Fig. 4*A*). 2) If reducing HEI10 dosage increases cross-over interference strength, then it could yield a stronger bias within two-cross-over chromosome groups toward those that create two bivalents (solution 2 in Fig. 4*A*). The above scenarios make specific predictions: In the first we expect a bias in LCO toward more four-chromosome groups having only two cross-overs relative to wild type, but no difference in outcome ratios within a cross-over number class. In the second, we would expect that for four-chromosome groups with two cross-overs, the ratio of 2B to 1T1U configurations should be higher in LCO than WT, while if only scenario 1 is relevant, there should be no difference.

To explore these scenarios, we studied correlations between cross-over number (estimated by counting chiasmata in metaphase I) and configurations of the four-homolog groups (Fig. 4*B* and Dataset S2). We first asked whether 2B configurations are indeed more frequent with lower cross-over numbers (Figs. 1 and 4). We observed that 88% of four-homolog groups that have 2 chiasmata have the diploid-like 2B configuration, whereas with more chiasmata the 2B configuration is never more frequent than 37% (independence chi-square, $P < 10^{-9}$ and $P < 10^{-17}$ for WT and LCO respectively; Fig. 4*B*). Then, we showed LCO neotetraploids have a higher frequency of four-homolog groups with only 2 chiasmata (~25% vs. ~9% in WT, Fig. 4*C*), corresponding to a general downward shift in the distribution of numbers of chiasmata per four-homolog group (Fig. 4*B*, goodness of fit chi-square $P < 10^{-73}$). These patterns suggest cross-over rate reduction in LCO neotetraploids increases frequency of diploid-like 2B configurations because it increases the frequency of four-chromosome groups with only two cross-overs. But is that all? The second scenario above makes the prediction that within the two-cross-over class, four chromosome groups should show a stronger bias favoring 2B configurations in LCO than WT (Solution 2, Fig. 4*A*). Our data show that four-homolog groups with two chiasmata do not differ in the proportion of 2B vs. 1T1U configurations between LCO and WT neotetraploids, suggesting no bias in *cis* vs. *trans* cross-over positioning (Fig. 4*B*, Fisher's exact test, $P > 0.488$), and thus solution 2 does not seem to operate here. We also did not observe LCO neotetraploids having higher 2B frequencies in any chiasmata number class (Fisher's exact test, $P > 0.999$ for all classes, adjusted with Holm–Bonferroni correction), suggesting there are no other substantial differences between LCO and WT neotetraploids beyond what can be explained by the shift toward lower cross-over number four-chromosome groups.

Overall, from this analysis, we conclude that the reduction of cross-over number is solely responsible for the observed increase in bivalent formation in LCO neotetraploids and any effect HEI10 reduction may have on interference is negligible for the observed increased 2B frequency. The slight increase in univalents can thus be explained by the observation that univalent-bearing configurations (1T1U and 1B2U) almost exclusively occur in 1- and 2-chiasmata four-homolog groups (Fig. 4*A*), which are more frequent in LCO neotetraploids.

Discussion

Progressive improvement of meiotic stability after WGD (cytological diploidization) correlates in most autopolyploid species with a reduction in multivalent frequency, particularly of trivalent plus univalent combinations (10, 24, 55). In most species, cytological diploidization is associated with decreased crossing-over and an increase in bivalent frequency at the expense of all types of multivalents. In a few species, reduced trivalent+univalent frequency is correlated instead with increased cross-over rates and higher quadrivalent frequency (reviewed in ref. 18). The latter apparent exception to the general trend that established polyploids have reduced multivalent rates, suggests that the primary target of selection against multivalents is in fact primarily the 1T1U configurations that give rise to unpartnered univalents (18). Even though it is being explored as a way of increasing neopolyploid fertility, the hypothesis that engineering reduced cross-over frequency could suffice to drive cytological diploidization by reducing multivalent formation rates in neopolyploids [(17, 23, 30, 31) for review ref. 18], has not been explicitly tested.

To test the hypothesis that there is indeed a simple association between cross-over rate and multivalent frequency in neotetraploids that we can manipulate, we generated lines that were *hei10* heterozygotes (*hei10-2*/+) and mutant for *taf4b*. Each mutant alone showed ~20% reduction in class I cross-overs, consistent with previous findings (42, 45), while the double-mutant lines had ~37% fewer class I cross-overs (33% of total cross-overs assuming 1.5 class II events per meiosis) relative to wild type, suggesting these mutations have an additive effect. Importantly, though many male meiocytes in these LCO lines had only 5 class I cross-overs (equal to the number of chromosomes), we did not observe any univalents, indicating they retain robust cross-over assurance. This 33% reduction of total cross-overs is maintained in LCO neotetraploids relative to wild-type neotetraploids. This observation also suggests that, given the very likely conservation of HEI10 dosage dependency across species (42, 56–58), it might be possible to leverage this system to further decrease cross-overs without producing univalents in multiple species. This could be exploited not only to stabilize meiosis in neotetraploids, but also in breeding programs that aim to minimize breakage of linkage blocks while meiosis is still functional (59).

We found a clear association in *A. thaliana* neotetraploids between a lower cross-over number per four-homolog group and increased likelihood of diploid-like bivalent formation. This was true especially for the smaller Chromosomes 2, 3 and 4; multivalent rates remained nearly unchanged for the large Chromosomes 1 and 5. This is consistent with a prediction made already in the late 1930s (30, 31) that shorter chromosomes have greater likelihood of forming bivalents in neoautopolyploid meiosis due to the positive correlation between chromosome length and cross-over number. This trend is also consistent with models for how HEI10 dosage affects cross-over rates and spacing, which has been explained recently with a "coarsening" model: HEI10 loads on chromosomes initially at similar levels per µm axis length, but as meiosis progresses, foci congeal by accumulating HEI10 from neighboring regions, leading to progressively wider spacing of larger and larger foci, the largest of which ultimately yield cross-overs in a pattern fitting with cross-over interference (39, 60). In our lines, HEI10 dosage is reduced, suggesting that the above trends are explained because the smaller chromosomes load less HEI10 simply because they are shorter. As a result, the smaller chromosomes should become even more likely in the mutant lines to have just single cross-overs, while the larger chromosomes may still accumulate sufficient HEI10 to make two cross-overs even in the LCO lines.

Our results show that reducing multivalent frequency by simply reducing cross-over numbers does work, but we found that it comes with a slight cost of an increase in frequency of univalents. This may in many cases be an acceptable trade-off, as the univalent rate remains low also in LCO neotetraploids. Initially this increase in univalents was somewhat surprising, since the double mutant

shows normal cross-over assurance and no univalents in the diploid state. However, our data suggest that the increase in univalents comes not from a loss of cross-over assurance specifically in the LCO neotetraploids but is rather a by-product of the increased frequency of four-chromosome groups having just two cross-overs total, which can lead either to a 2B or 1T1U outcome (Fig. 4). It seems to be the increase in 1T1U configurations that leads to the increase in univalents in the LCO neotetraploids relative to wild type. Why is this uptick in univalents not observed in established polyploids in other species? Some hints come from work in the closely related natural autotetraploid, *A. arenosa*. While evolved autotetraploids of *A. arenosa* have reduced cross-overs relative to neotetraploid *A. arenosa*, this reduction correlates with decreased multivalent and univalent formation rates (15). Most importantly for interpreting the results here, in four-homolog groups that receive only two cross-overs, evolved *A. arenosa* autotetraploids show a much stronger bias toward the 2B configuration (and away from 1T1U) than the neotetraploids. This bias seems to arise largely from an increase in cross-over interference, and perhaps also an additional contribution from a decrease in axis length (increasing effective interference as a proportion of a chromosome along which it can act), which helps ensure that cross-overs are not formed on opposite sides of a partner switch event (15) (Fig. 4*A*). Interestingly, in *A. arenosa*, these adaptations seem to be genetically separable, as described below.

The above suggests that there may be at least two separate adaptations to autopolyploid meiosis: one which reduces multivalent frequency and a second which reduces the frequency of univalents that arise as a by-product. Importantly we show here that the second is not an automatic consequence of the first. Previous genome scans for adaptation to polyploidy in *A. arenosa* identified several meiosis genes showing evidence for selection (61, 62). In functional follow-up on three of them, we have shown that reducing multivalent frequency and reducing univalent frequency may indeed be genetically separable adaptations. A derived (evolved tetraploid) allele of the meiosis-specific cohesin subunit REC8 is associated in tetraploids with a significantly lower rate of univalent production, but has no effect on multivalent rate or cross-over number (34). In contrast, derived alleles of the axis proteins ASY1 and ASY3 have a significant effect on reducing multivalent formation rates, but *increase* univalent frequency in the absence of the tetraploid allele of REC8, suggesting these may even be to some extent antagonistic adaptations (33, 34). Derived alleles of ASY1 and ASY3 also lead to increased bias toward the 2B configuration (33), and all three genes lead to reduced axis length, which may also contribute to the bias to 2B of two-cross-over four-chromosome groups (34). It is intriguing that even though HEI10 is implicated in cross-over interference via the coarsening model, our results suggest that reducing HEI10 dosage, which is predicted to increase cross-over spacing, does not solve the 2B/1T1U bias challenge in the neotetraploid *A. thaliana* lines. Together, these data suggest that the evolutionary path to meiotic stabilization in tetraploids likely involves at least two components: 1) a reduction in overall multivalent frequency, and 2) a bias toward 2B outcomes on LCO four-chromosome groups achieved by stronger cross-over interference (15).

In aggregate our results do show that in principle engineering genotypes with reduced cross-over rates can work for reducing the frequency of multivalents in neopolyploids. Another important finding, however, is that reducing multivalents is not sufficient on its own to rescue low neopolyploid fertility, which has implications for both natural and engineered systems. The extremely low frequency of univalents in neotetraploids (both in LCO and WT) is unlikely to suffice on its own to explain the lack of fertility rescue in LCO relative to WT neotetraploids. This means there is something more, but what this additional factor or factors is/are remains mysterious, though the existence of "something else" was already proposed 75 y ago (16). This may raise the question, do multivalents matter at all? We believe that the answer is yes. There is strong evidence that selection has acted to reduce multivalent frequency in the majority of evolved tetraploids, including *A. arenosa*, where we have identified evolved alleles of two genes that together help reduce multivalent frequency (33). These trends strongly support the idea that multivalents do matter, even if they are not the entire problem. Solving the multivalent problem, for which we show here reducing recombination rate is sufficient, will thus likely be an important part of a multi-component solution to neopolyploid fertility challenges.

Material and Methods

Plant Material. We used the previously described T-DNA insertion lines SALK_014624 (N514624) and SALK_025468 (N525468) carrying the mutant alleles *hei10-2* (40) and *taf4b-2* (45), respectively, in *A. thaliana* Col-0 background. To produce neopolyploids we treated the apical meristem of 14-d-old seedlings with 0.05% colchicine (Sigma C9754) diluted in sterile water with 0.05% Silwet-77 (Anawa 30630216). We identified neopolyploid branches by flow cytometry performed on young flowers.

Genotyping. For genotyping, we used the previously described PCR primers (40, 45) given in *SI Appendix*, Table S1. For *HEI10* genotyping, we used the primers hei10-2-F1 + hei10-2-WT-R to detect the WT allele and hei10-2-WT-R + hei10-2-mut-R to identify the mutant allele in PCRs with a T_M of 60 °C. For TAF4b genotyping, we used the primers taf4b-2-LP + taf4b-2-RP to detect the WT allele and LBb1.3 + taf4b-2-RP to identify the mutant allele in PCRs with a T_M of 60 °C.

Seed Count Analysis. Before counting seed sets of colchicine-treated plants, we identified truly neotetraploid siliques by flow cytometry performed in the petiole of the silique. We only used a maximum of 12 siliques from the main inflorescence and discarded the three that formed first. After registering the ploidy of each silique, we bleached them in 70% ethanol during 1 wk and then we counted the number of developing seeds. Seed count data is reported in Dataset S3.

Pollen Viability Analysis. For pollen viability analysis, we only used colchicine-treated plants. After genotyping, we performed flow cytometry in petioles to identify truly neotetraploid and diploid flowers. Then, we use commercial Alexander staining (Morphisto 13441) to stain either released pollens or anthers that were imaged using Leica Thunder Imager 3D Tissue epifluorescence microscope. Details about the pollen viability data are included in Dataset S4.

Meiotic Spreads. Prior to cytological analyses we performed spreads of meiotic chromosomes following the protocol in ref. 51 for FISH, or in ref. 63 for immunolocalization, with minor modifications. Briefly, we fixed flower buds in 3:1 ethanol:acetic acid for at least 48 h and for at most 7 d for immunostaining. We sorted flower buds by size and incubated them in of enzyme mixture (0.3% cellulase, 0.3% pectolyase, 0.3% cytohelicase in 10 mM citrate buffer) at 37 °C in a moist chamber for 90 min for immunostaining or 150 min for FISH. Then, we crushed ~6 buds with a brass rod in of 60% acetic acid on a slide. Next, after placing the slide on a heat block at 45 °C for immunostaining or 42 °C for FISH, we macerated with a needle for 1 min. Then, we added 3:1 ethanol: acetic acid around the macerate to fix the sample and washed the slide two more times with 3:1 ethanol: acetic acid. We finally air-dried the slides and mounted them with DAPI in Vectashield mounting medium. Chiasmata counting data in diploids are detailed in Dataset S2.

Immunolocalization. To immunolocalize HEI10 in our chromosome spreads, we first selected slides containing enough cells in late prophase I (mostly diakinesis but also some diplotene). Then, we followed the protocol described in ref. 63. Briefly, we performed a 45-s microwave treatment at 850 W in our slides containing spreads (from recently fixed material) immersed in citrate buffer pH 6. Immediately after microwave, we washed the slides in PBS-T (0.01% Tween-20 diluted in PBS buffer) for 5 min and then incubated in presence of an antibody

against *A. thaliana* HEI10 (Biomatik) raised in rabbit serum in dilution 1:200 overnight. Then, after 3 washes in PBS-T we incubated using Alexa488-anti-rabbit secondary antibody (Thermo Fisher Scientific A-11034) and mounted the slides. Cells were visualized and imaged using Leica Thunder Imager 3D Tissue epifluorescence microscope. HEI10 foci counting data are detailed in Dataset S1.

FISH. For FISH, we use the pTA71 and pCT4.2 probes containing the 45S and 5S rDNA, sequences, respectively, following the protocol in ref. 51. Briefly, after selecting chromosome spreads containing enough cells in metaphase I, we incubated them in in saline sodium citrate (SCC) buffer, and digested in pepsin. Then, we washed again in SCC and fixed the material in paraformaldehyde and dehydrated in increasing concentrations of ethanol followed by air-drying. We next performed DNA denaturation of the sample and the probes at 72 °C and allowed them to hybridize over night at 37 °C in a moist chamber overnight. Then, after washes in 50% formamide and SCC at 45 °C, we incubated the samples with the secondary antibodies (anti-digoxigenin-FITC and streptavidin-Cy3) at 37 °C for 1 h in darkness before mounting the slides with DAPI in Vectashield mounting medium. Cells were visualized and imaged using a Nikon Eclipse 90i microscope equipped with the Hamamatsu CCD camera and controlled by the NIS-Elements Advanced Research software. Cytological analysis by FISH data in neotetraploids are detailed in Datasets S5 and S6.

Data Generation and Analysis

Chiasmata count and metaphase I configurations scoring were performed according to the criterion described for diploids and tetraploids in refs. 48 and 51, respectively. In all cases, the scoring was done completely blindly with previously randomized and anonymized images using the Blind Analysis Tool ImageJ plugin.

For two-groups statistical comparisons of quantitative data (such as per cell number of chiasmata, HEI10 foci, or 2B configurations) we used either *t* test or Mann–Whitney *U*, depending upon whether the compared samples passed a normality Kolmogorov–Smirnov test (in Dataset S7). When independent pair-wise tests were performed within the same family of comparisons (e.g., comparing individual chromosomes in WT vs. LCO context, without comparing different chromosomes between each other) we treated each case as an individual two-groups comparison suitable for *t* test or Mann–Whitney *U*, but we did apply the corresponding Holm–Bonferroni *P* value correction (as they belonged to the same family of comparisons). For multiple group comparison test, since in all the cases at least one group did not pass the normality Kolmogorov–Smirnov test, we always used nonparametric Kruskal–Wallis test with Dunn's correction. To compare seed yield, we did not pool treated and untreated diploid plants, since they had very different distributions and SDs. To statistically compare the frequencies of categories, such as types of pollens, metaphase I associations, or four-homolog groups configurations, we used independence and goodness of fit tests. These included chi-square tests, when less than 20% of the expected values equaled 5 or less, whereas Fisher's exact test was used when this condition was not fulfilled. When multiple nonindependent chi-square or Fisher's exact tests were performed within the same family of comparisons we used Holm–Bonferroni correction to adjust the *P*-value. Statistical tests were performed using Graphpad Prism (*t* test, Kruskal–Wallis, Mann–Whitney *U*, and F test for variances), Microsoft Excel (Chi-Square), or R (Fisher's exact test). Detailed information on statistical tests is provided in Datasets S7 and S8.

Data, Materials, and Software Availability. All image data used in this study are available freely from the ETH Research Collection https://doi.org/10.3929/ethz-b-000605099 (64).

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