



Genome balance and dosage effect drive allopolyploid formation in *Brassica*

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Polyploidy is a major evolutionary force that has shaped plant diversity. However, the various pathways toward polyploid formation and interploidy gene flow remain poorly understood. Here, we demonstrated that the immediate progeny of allotriploid AAC *Brassica* (obtained by crossing allotetraploid *Brassica napus* and diploid *Brassica rapa*) was predominantly aneuploids with ploidal levels ranging from near-triploidy to near-hexaploidy, and their chromosome numbers deviated from the theoretical distribution toward increasing chromosome numbers, suggesting that they underwent selection. Karyotype and phenotype analyses showed that aneuploid individuals containing fewer imbalanced chromosomes had higher viability and fertility. Within three generations of self-fertilization, allotriploids mainly developed into near or complete allotetraploids similar to *B. napus* via gradually increasing chromosome numbers and fertility, suggesting that allotriploids could act as a bridge in polyploid formation, with aneuploids as intermediates. Self-fertilized interploidy hybrids ultimately generated new allopolyploids carrying different chromosome combinations, which may create a reproductive barrier preventing allotetraploidy back to diploidy and promote gene flow from diploids to allotetraploids. These results suggest that the maintenance of a proper genome balance and dosage drove the recurrent conversion of allotriploids to allotetraploids, which may contribute to the formation and evolution of polyploids.

genome balance | dosage effect | triploid bridge | allopolyploid | gene flow

Polyploidy has played a major role in plant evolution and speciation (1). Ref. 2 Estimated that change in ploidy accounts for 35% of speciation across vascular plants (2). Notably, all modern angiosperms have experienced at least one round of polyploidy in their evolutionary history (3–5). However, despite the importance of polyploid evolution, the mechanisms leading to the formation and establishment of polyploidy remain poorly understood (6, 7).

Several cytological mechanisms have been described to induce polyploid formation in plants (8, 9). The triploid bridge pathway, unilateral sexual polyploidization associated with unreduced gamete formation in diploid populations (10), is regarded as a major route to plant polyploidization and speciation (9, 11). However, triploids are often thought to be meiotically irregular, and the generated aneuploid gametes usually are sterile or have low fertility (9, 12, 13). Until now, the mechanism by which triploids can produce polyploids has remained elusive, although different pathways have been proposed (8, 9). Several studies in plants support the hypothesis that triploids might produce low-frequency euploid gametes that ultimately result in the formation of tetraploids (9, 14–17). Some studies have proposed that triploids could generate unreduced gametes (9, 18, 19), which may contribute to tetraploid formation (8, 20, 21). To better understand the process of polyploid formation from the triploid bridge, direct evidence is required to understand the associated pathways, intrinsic mechanisms, and rates of polyploid formation (9, 19, 20).

A reproductive barrier between new polyploids and their diploid progenitors may impact polyploid establishment and persistence (22). Triploids, the interploidy hybrids of tetraploids and their diploids, act as an important reproductive barrier and the main force underlying polyploid persistence (19, 23, 24). Newly formed polyploids have a high degree of immediate postzygotic reproductive isolation from their progenitors arising from embryo abortion by genomic imbalance in the endosperm (i.e., triploid block) after the crossing of plants with different ploidal levels and/or produced by low viability and fertility of interploidy hybrids (10, 25). However, different hybridization abilities in both directions in interploidy crosses between polyploids and diploids have been reported in different natural populations of plants (24, 26). In addition, interploidy hybrids from different taxa exhibit various levels of fertility ranging from sterility and semisterility to mildly reduced fertility (24, 27). These results demonstrated that the reproductive barrier of

Significance

Polyploidy has played a major role in the evolution of flowering plants, yet little is known about the genetic mechanisms that contribute to the formation and stability of polyploids. In this study, we discovered that allopolyploid formation via allotriploids was a feasible and gradual process using aneuploids as intermediates and driven by genome balance and dosage effects. Interploidy hybrids between allotetraploid *B. napus* and diploid *B. rapa* ultimately developed into new allopolyploids, indicating that the reproductive barrier against allotetraploidy reversion to diploidy was enhanced while gene flow from diploids to allotetraploids was promoted by this process. Our findings provide a mechanism of polyploid formation and persistence.

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polyploids and diploids could be overcome to a certain extent (19), and hybridization flows (or pollen flows) exist between diploids and polyploids in many species (9, 26, 28). However, since information about the final fates of interploidy hybrids was never evaluated in most previous studies, the contradictory roles of interploidy hybrids in the reproductive barrier and genetic bridge between polyploids and their diploid progenitors remain unclear.

Brassica napus ($AACC$, $2n = 4x = 38$) is a relatively young allopolyploid resulting from hybridization between two progenitor species, *Brassica rapa* (AA , $2n = 2x = 20$) and *Brassica oleracea* (CC , $2n = 2x = 18$) (29). The ease of ploidy manipulation and interspecific hybridization, the existing suite of molecular cytogenetic techniques, and the availability of reference genome sequences make *B. napus* an ideal model species for investigating the consequences and mechanisms of de novo allopolyploidization (30–33). In this study, using cytogenetic techniques to unambiguously identify every chromosome, we investigated the karyotypes and phenotypes of the offspring of AAC allotriploids in successive generations. The results allowed us to discover the pathway and mechanism of allopolyploid formation via triploids.

Results

Allotriploids of *Brassica* Are Prone to Produce Aneuploid Progeny Harboring More Than One Complete Set of C Chromosomes.

Allotriploids of *Brassica* (AAC) were produced by four kinds of cross methods to imitate different origins of triploids (Fig. 1). The chromosomal compositions of all the generated allotriploids were verified by karyotyping using the multicolor fluorescence in situ hybridization (FISH) method developed by ref. 34 (SI Appendix, Fig. S1A). This karyotype analysis allows unambiguous identification of all the mitotic chromosomes and their corresponding homoeologs in *B. napus*.

Self-fertilized AAC allotriploids were not completely sterile and generated an average of 60 viable seeds per plant. The morphological phenotype of allotriploids was similar to that of allotetraploid *B. napus*. However, the immediate progeny of allotriploids included plants exhibiting various phenotypes, such as altered leaf

shapes and colors, dwarfism ranging from subtle to extreme, and changes in flowering time and fertility (SI Appendix, Fig. S1B).

To investigate the genome structure and chromosome organization of the immediate progeny of AAC allotriploids (AAC, $2n = 3x = 29$), the detailed chromosomal compositions of 97 plants in the F_2 generation produced by self-fertilization from 14 individuals belonging to four kinds of allotriploids were investigated by karyotyping analysis and/or genome resequencing (Fig. 1 and SI Appendix, Table S1).

The immediate progeny of allotriploids showed a wide range of chromosome numbers from 26 to 61 (Fig. 2A). Most F_2 plants inherited approximately 29 to 36 chromosomes from their AAC parents. The chromosome numbers in the F_2 plants exhibited asymmetrical binomial distributions with a maximum frequency of 15.5% and a peak at 32 chromosomes. The observed distribution of chromosome numbers in the F_2 population deviated from the theoretical distribution (homologous A chromosomes equally and C chromosomes randomly segregate into gametes, and random fusion of male and female gametes in the absence of selection), with a shift toward gaining three more chromosomes and only a few individuals carrying fewer than 29 chromosomes (Fig. 2A). This tendency for an increasing chromosome number was detected in the F_2 plants of allotriploids with different origins (Fig. 2A). This skewed distribution indicated that F_2 plants with 29 to 36 chromosomes were more viable and that the F_2 population experienced selection.

To determine the type of selection experienced by the viable progeny derived from allotriploids, we analyzed the chromosomal compositions of 92 plants (Dataset S1) in the immediate progeny of AAC allotriploids and found that all the karyotyped immediate progeny were aneuploids. Based on ploidal level, we divided the F_2 plants into three classes: near-hexaploid, near-pentaploid, and near-triploid (Figs. 2B–E and 3A).

One near-hexaploid with 61 chromosomes was detected in the F_2 population. This plant had four complete sets of A chromosomes and two complete sets of C chromosomes plus one more A6 chromosome and two more C3 chromosomes (Fig. 2B). The genome of this plant was unstable since the chromosome number

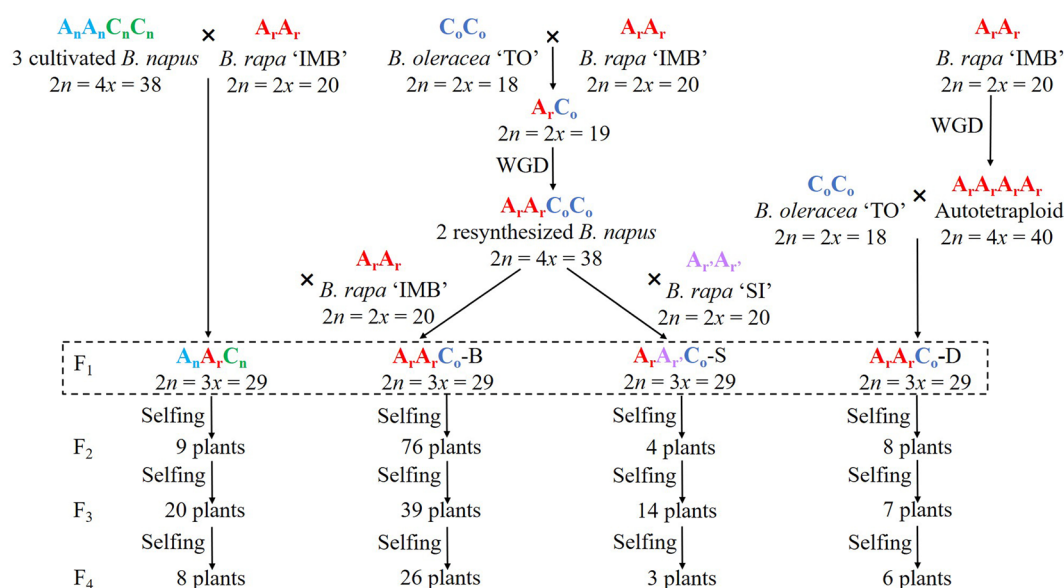


Fig. 1. Scheme of generating different allotriploids of AAC *Brassica* and detailed information about the production of the progeny of allotriploids by self-fertilization. $A_nA_rC_n$ was generated by interploidy hybridization between natural *B. napus* and *B. rapa*. $A_rA_rC_o-B$ (backcross) was obtained by backcross between resynthesized *B. napus* and *B. rapa* IMB. $A_rA_rC_o-S$ (SI) was obtained by interploidy hybridization between resynthesized *B. napus* and a different *B. rapa* SI. $A_rA_rC_o-D$ (double) was generated by crosses between genome-doubled *B. rapa* IMB and diploid *B. oleracea* TO1000.

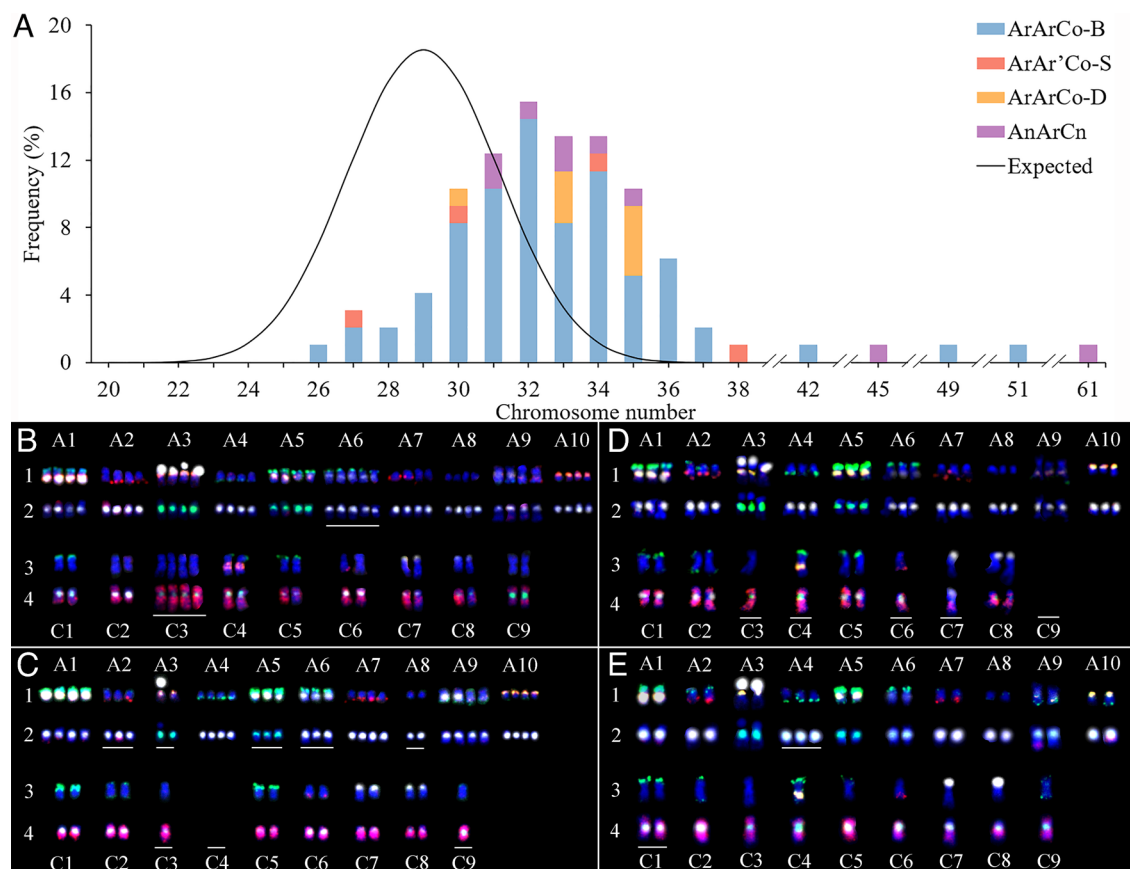


Fig. 2. Chromosome number distribution and karyotype analyses of the immediate progeny of AAC allotriploids. (A) The F_2 population showed a wide range of variation in chromosome number. The theoretical distribution (curved line) of chromosome numbers expected from random chromosome segregation in both male and female gametes of triploids in the absence of selection is a binomial distribution with a peak at 29 chromosomes. (B–E) For karyotype analysis, the A chromosomes are shown in lanes 1 and 2, and the C chromosomes are shown in lanes 3 and 4. The first round of FISH included 45S rDNA (white), 5S rDNA (yellow), BAC clone KBr072L17 (green), and KBrH092N24 (red) probes, and the hybridization results are shown in lanes 1 and 3. The second round of FISH included CentBr1 (white), CentBr2 (green), and BAC BNIH 123L05 (red) probes containing C genome-specific repeated sequences, and the hybridization results are shown in lanes 2 and 4. (B) One near-hexaploid derived from the $A_6A_6C_6$ -S allotriploid contains 61 chromosomes. (C) The karyotype of one branch of the near-hexaploid shows a change to 47 chromosomes and many chromosome deletions (underlined). (D) Plant 19N001-2 is a near-pentaploid containing three complete sets of chromosomes from the A genome and an incomplete set of chromosomes from the C genome. (E) Plant 500IMB-A-45 is a near-triploid, with two complete sets of chromosomes from the A genome and one complete set of chromosomes from the C genome plus one additional A4 chromosome and one additional C1 chromosome.

of one of its branches changed to 47 (Fig. 2C). Five individuals containing 38 to 51 chromosomes were observed, and four of them had three complete sets of chromosomes from the A genome plus different numbers of C chromosomes (Fig. 2D), suggesting that they might be near-pentaploids formed by the merging of two gametes containing one set and two sets of A chromosomes. The third class of F_2 plants was near-triploid (or near-tetraploid, hereafter referred to as near-triploid), including 86 plants, which had chromosome numbers from 26 to 37, and most of them contained two complete or near-complete chromosome sets from the A genome, as well as different numbers of C chromosomes (Fig. 2E). According to the C chromosomal composition, we divided the near-triploid plants into three types. The first type of near-triploids contained one complete set of chromosomes from the C genome plus additional C chromosomes (hereafter referred to as complete C near-triploids; *SI Appendix, Fig. S2A*). Fifty-seven individuals, representing 61.96%, of F_2 plants were complete C near-triploids (Fig. 3D), which indicated that a complete set of C chromosomes was important for the survival of the immediate progeny of allotriploids. The second type of near-triploids was compensated near-triploids, which contained one complete set of C chromosomes except for one C chromosome but an extra corresponding homoeologous A chromosome leading to dosage

compensation (35) (*SI Appendix, Fig. S2B*). A total of eight compensated near-triploid plants, which accounted for 8.7% of the total F_2 plants, were detected (Fig. 3D). These compensated near-triploids may have been formed by the merging of a gamete containing nondisjunct A homologs with other gametes. The third type of near-triploids was imbalanced near-triploids, which had an incomplete set of C chromosomes and contained at least one dosage-imbalanced chromosome with only two copies of homoeologs (*SI Appendix, Fig. S2C*). Twenty-one individuals, representing 22.83% of the F_2 plants, were imbalanced near-triploids (Fig. 3D). Only five individuals, representing 5.4% of the F_2 plants, had fewer than 29 chromosomes, which was significantly lower than the theoretical frequency of 40.75% assuming no selection (Chi-square test, $P = 2.98 \times 10^{-9}$).

Thus, our results demonstrated that in the immediate progeny of AAC allotriploids, there was selection in favor of individuals containing at least one complete set of chromosomes from the C genome, which led to the observed chromosome number distribution deviating from the theoretical distribution.

Fertility of the Immediate Progeny of Allotriploid AAC Brassica.

The fertility of F_2 plants was estimated by the percentage of pollen viability and the total seed yield. The near-hexaploid had a high

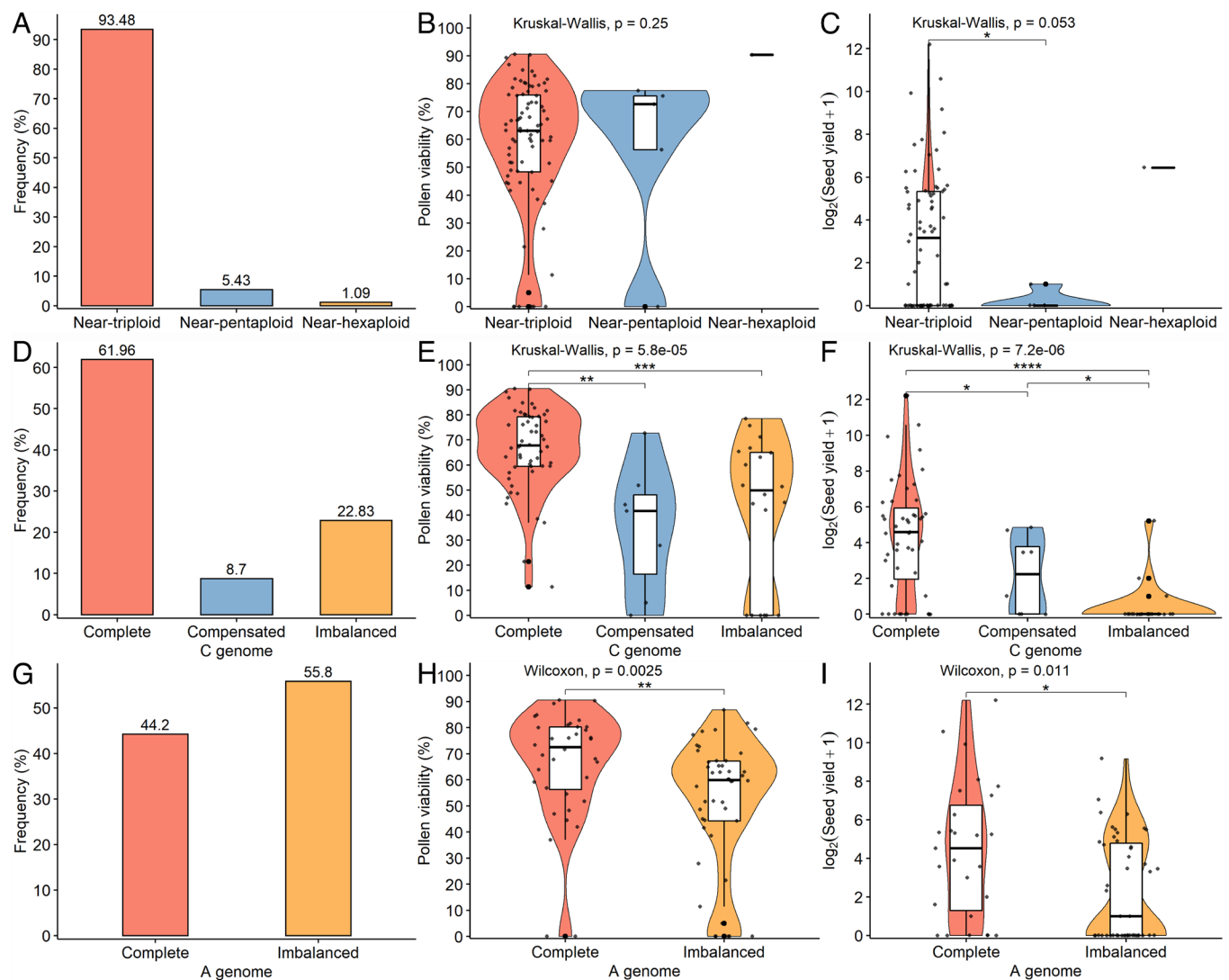


Fig. 3. Fertility of the immediate progeny (F₂ population) of allotriploid *Brassica*. (A) The frequency of the three different classes (near-triploid, near-pentaploid and near-hexaploid) of aneuploids. (B) The pollen viability of different classes of aneuploids. (C) Seed yield of different classes of aneuploids. (D) The frequency of three different types (complete, compensated, and imbalanced) of near-triploids, grouped by the chromosomal composition of the C genome. (E) The pollen viability of three types of near-triploids. (F) The seed yield of three types of near-triploids. (G) The frequency of near-triploids containing genome-balanced (two complete sets of chromosomes) and imbalanced (two incomplete sets of chromosomes) chromosomes from the A genome. (H) The pollen viability of plants with a balanced or an imbalanced A genome. (I) The seed yields of plants with a balanced or an imbalanced A genome. Statistical significance was calculated with the Kruskal-Wallis test in B–F and with the Wilcoxon test in H and I. Asterisks represent statistically significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$) between the indicated groups. The original seed yield is logarithmically transformed ($\log_2(\text{seed yield} + 1)$) in C, F, and I.

pollen viability of 90.3% and produced 86 seeds (Fig. 3 B and C), while one of its branches with 47 chromosomes had lower pollen viability (71.6%) and produced no seeds (Dataset S1). The five near-pentaploids in the F₂ population showed variation in pollen viability from zero to 77.5%, and only one plant generated one seed.

The near-triploids (86 plants) had an average pollen viability of 57.8%, which was similar to that (56.4%) of near-pentaploids (Fig. 3 B and Dataset S1). The near-triploids showed wide variation in seed set from zero seeds in some plants to 4,722 seeds in plant 19N003-11, which contained a complete set of chromosomes of *B. napus* except for monosomic C3 and C8. Fifty-two percent of the near-triploids produced seeds and, on average, the near-triploids yielded 130.9 seeds per plant.

To understand whether the chromosomal compositions from the C genome impacted phenotypes and fertility, we comparatively analyzed the pollen viabilities and total seed yields of the three different types of near-triploids. We found that the complete C

near-triploids had significantly higher pollen viabilities and seed yields than the compensated near-triploids and imbalanced near-triploids (Fig. 3 E and F). According to the seed yield data collected from 47 individuals of complete C near-triploids, 37 (78.7%) individuals generated seeds, with an average number of 203.7 seeds per plant. Five of the eight compensated near-triploids produced a few seeds and generated, on average, 9.3 seeds per plant. Most of the imbalanced near-triploids (85.7%, 18 plants) were sterile, and the other three individuals showed very low seed sets with an average of 2.1 seeds per plant (Dataset S1). These results demonstrated that a complete set of C chromosomes and dosage compensation had significant positive impacts on the fertility of the F₂ plants.

About 44.2% (38 plants) of the near-triploids among the F₂ plants had exactly two complete sets of A chromosomes, while the remaining 55.8% (48 plants) of near-triploids contained imbalanced A genomes carrying monosomic or trisomic A chromosomes. The former group had significantly higher median pollen

viabilities and median seed yields than the latter group (Fig. 3 *G–I*), which indicated that an imbalanced A genome also had a detrimental effect on fertility.

Self-Fertilized Allotriploids Developed into Allotetraploids in Several Generations by Gradually Increasing Their Chromosome Numbers. To investigate the chromosomal composition and fate of the self-fertilized allotriploids, we comparatively analyzed the karyotypes of the progeny from the F_2 to F_4 generations (Fig. 4 and [Datasets S1–S3](#)). In the F_3 generation, 80 plants generated directly from the seeds of 55 different plants in the F_2 generation, which were broadly representative ([SI Appendix, Materials and Methods](#)), were used for karyotypic and phenotypic studies. The chromosome number of the F_3 population varied widely from 30 to 48, and most F_3 plants had 33 to 39 chromosomes (Fig. 4*C*). The chromosome numbers in the F_3 plants exhibited an asymmetrical binomial distribution, with the main peak at 37 chromosomes. Compared with the observed chromosome number distribution in the F_2 population, that in the F_3 population was shifted toward five more chromosomes.

Seventy-six plants in the F_3 generation were karyotyped, and chromosomal composition data were obtained. Among them, four near-pentaploids, four near-hexaploids, and 68 near-tetraploids were detected. Among the 76 plants, 90.8% contained at least one or up to two complete sets of C chromosomes, which further indicated strong selection in favor of individuals containing a balanced C genome. In addition, another four individuals among the F_3 plants yielded data for chromosome number but

chromosomal composition was not determined ([Dataset S2](#)). We found a total of eleven F_3 plants containing 38 chromosomes. Among them, one plant had a perfect set of *B. napus* chromosomes, and five plants were compensated allotetraploids with one chromosome substitution between homoeologs (35).

In the F_4 population, forty-two plants generated by self-fertilization from 28 randomly selected plants in the F_3 generation were karyotyped. The F_4 plants contained 35 to 40 chromosomes, with the most common chromosome number being 38 (Fig. 4*D*). Fifteen (35.0%) F_4 plants had 38 chromosomes; among them, five individuals were allopolyploids with a perfect chromosome set from *B. napus* ([SI Appendix, Fig. S3](#)), while another four individuals were compensated allopolyploids. All the AAC allotriploids produced by four different cross methods or with different donors for their A and C genomes were able to generate new complete (or compensated) allotetraploid *B. napus* in the F_4 generation, indicating that triploid origins have no detectable impact on genetic features studied ([Dataset S3](#)). Similar to the pattern in the F_3 population, most (87.5%) of the F_4 plants had gained at least one or up to two complete sets of C chromosomes, along with two complete (or near-complete) sets of A chromosomes ([Dataset S3](#)).

In the offspring of AAC allotriploids, we observed that C chromosomes that were lost were often compensated for by the gain of homoeologous A chromosomes, particularly for homoeologous chromosome sets A1/C1 and A2/C2. Among the 193 plants karyotyped from the F_2 to F_4 generation, 28 and 18 individuals gained one or two extra A1 and A2 chromosomes, respectively ([SI Appendix, Table S2](#)). There was strong bias for extra A chromosomes to

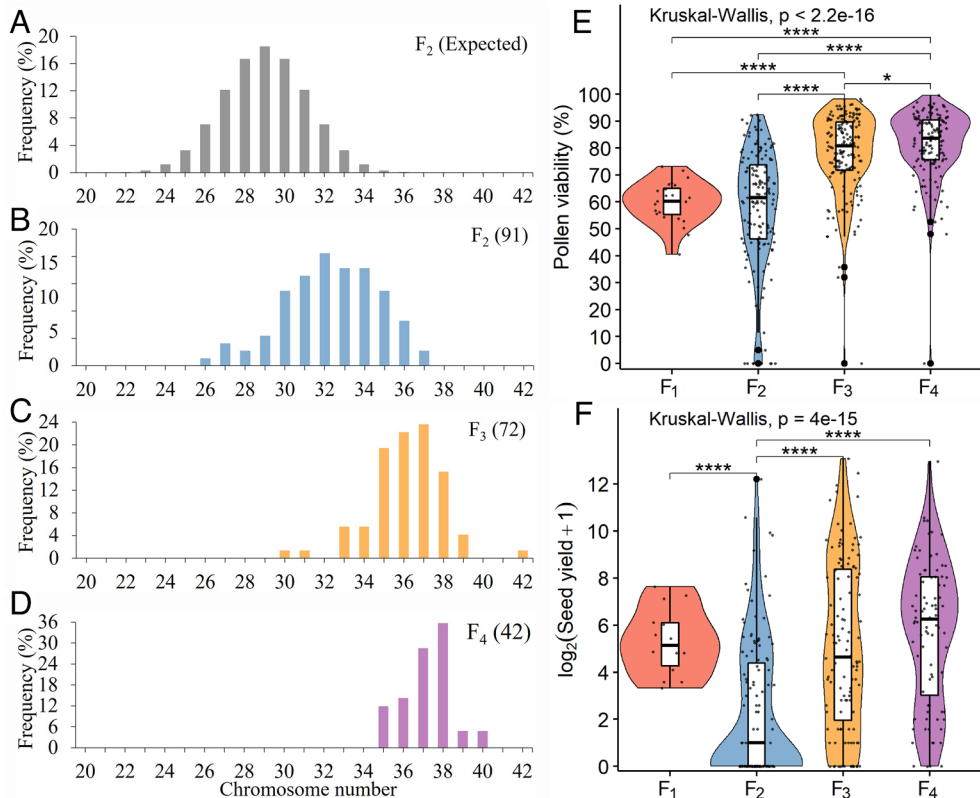


Fig. 4. Chromosome number distributions and comparison of the fertilities of the self-fertilized progeny in different generations of AAC allotriploids. (A) The expected chromosome number distribution is a symmetrical binomial distribution with a peak at 29 chromosomes. (B) The observed chromosome number distribution of the 91 near-triploids in the F_2 generation. (C) The observed chromosome number distribution of the 72 plants in the F_3 population. The plants used for chromosome number distribution analyses excluding near-hexaploids and near-pentaploids in *B–D*. (E) Comparison of pollen viabilities from F_1 to F_4 generation. (F) Comparison of seed yields from F_1 to F_4 generation. The original seed yield was logarithmically transformed ($\log_2(\text{seed yield} + 1)$). Statistical analyses were completed with the Kruskal–Wallis test. Asterisks represent statistically significant differences ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$) between the indicated groups.

accompany missing C homoeologous chromosomes (Chi-square test, $P = 1.1 \times 10^{-28}$ for A1/C1, $P = 3.11 \times 10^{-36}$ for A2/C2; *SI Appendix, Table S2*).

These results demonstrated that AAC allotriploids readily developed into allopolyploids after 2 to 3 generations of self-pollination by gradually increasing their number of C chromosomes under strong selection against aneuploid individuals with more imbalanced chromosomes from the A and C genomes.

The Fertilities of the Allotriploid Progeny Increased across Generations. The fertilities, including the pollen viability and total seed yield, of the progeny of AAC allotriploids were comparatively analyzed from the F₁ to F₄ generation (*Datasets S4 and S5*). Compared with that of AAC allotriploids, the median frequency of pollen viability gradually but significantly increased from the F₂ to F₄ generation (Fig. 4E). The median total seed yield also gradually but significantly increased from the F₂ to F₄ generation (Fig. 4F). In the F₄ generation, the average seed yield reached 294.3 seeds per plant, indicating that most F₄ plants were fertile. In particular, some individuals that had chromosome numbers consistent with those of allopolyploid *B. napus* or dosage-compensated allopolyploids in the F₃ and F₄ generations showed recovered fertility matching that of resynthesized *B. napus* (35).

In previous studies, the karyotypes of resynthesized *B. napus* were unstable in late generations, and the chromosome number remained balanced at approximately 38 with a high frequency of homoeologous shuffling (35, 36). Since the chromosome number distributions and chromosomal compositions in the F₄ population were similar to those of resynthesized *B. napus* (35–37), we did not further extensively karyotype the progeny of allotriploids after the F₄ generation.

Gene Flow from Diploids to Allopolyploids Using Triploids as a Bridge. The transmission of A chromosomes from natural A_nA_rC_n-M allotriploids (interploidy hybrids between natural *B. napus* Marnoo-DH and *B. rapa* IMB) to their self-fertilized progeny was assessed by genome sequencing using three individuals from the F₄ and F₅ generations, which exhibited karyotypes similar to those of *B. napus* (Fig. 5 A–C and *SI Appendix, Fig. S3*). Given that homologous A chromosomes paired and segregated normally in A_nA_rC_n-M allotriploids (*SI Appendix, Fig. S4*), heteromorphic sequences (or chromosomes) present in IMB218 (A_r) or Marnoo (A_n) would be transmitted with the same chance to the progeny in the absence of segregation distortion or selection.

Introgression analysis of the plant 20N066-2, an allotetraploid from the F₄ generation, showed that the main A1, A4, A6, and A10 chromosomes, as well as different sizes of fragments in other chromosomes, came from the diploid parent *B. rapa* (Fig. 5D). Large chromosome fragments from *B. rapa* were also observed in plants 19A051 and 20N079-2 from the F₄ and F₅ generations, respectively (Fig. 5 E and F). These results demonstrated that genome sequences from the diploid *B. rapa* were transferred to the newly formed allotetraploids. In addition, reciprocal crosses between the newly formed allotetraploids (20N079-2 and its progeny) and natural *B. napus* (Marnoo-DH) produced 16 to 24 seeds ($n = 9$; mean of 18.7 ± 2.6) per pollinated flower, demonstrating high crossing abilities of the triploid-derived allotetraploid with natural *B. napus*.

Discussion

Triploidy Is a Mediator of the Origins of Different Ploidy Cytotypes. Triploids are often expected to be meiotically irregular, resulting in sterility or very low fertility (9, 12, 13), often regarded

as an evolutionarily dead end. However, a survey of articles published in the past (9) revealed that both autotriploids and allotriploids are often semifertile and may contribute to tetraploid formation.

In this study, we demonstrate that the immediate progeny of allotriploid *Brassica* includes a swarm of aneuploids with a wide range of genome contents, which is consistent with cytometric observations from autopolyploid *Arabidopsis* (24) and allopolyploid *Brassica* (38). Pentaploids and hexaploids have been observed among the progeny of open-pollinated diploids and self-hybridizing triploids in many systems, including ferns, *Triticum*, and *Nicotiana* (39–44). Our results further demonstrate that allotriploid *Brassica* can generate offspring with different ploidal levels from near-triploidy to near-hexaploidy.

The Pathway from Triploid to Tetraploid. Several hypotheses have been proposed to explain how triploids develop into tetraploids (8, 9, 12). The most accepted hypothesis proposes that triploid individuals often generate a few normal gametes, including haploids, diploids, and triploids (14, 16, 17). Polyploids were assumed to arise directly by sexual reproduction via conjugation between haploid and triploid gametes or between diploid and diploid gametes derived from selfing or outcrossing (9, 19, 45). Another hypothesis proposes that unreduced gametes produced by triploids facilitate allopolyploid formation (8, 12). Evidence from autopolyploid *Arabidopsis* showed that autotriploids gave rise to a swarm of aneuploid progeny and then stabilized at either the diploid or tetraploid level, which indicated that triploids could function as bridges between euploidy types (24, 46).

In this study, we not only show that allotriploid *Brassica* can act as a bridge in allopolyploid formation but also find that the formation of allopolyploids via allotriploids is a gradual process involving several generations of aneuploids as intermediate steps. Limited by the number of F₂ plants karyotyped, our results do not support the hypothesis that allotetraploid formation via triploids occurs directly through the fusion of euploid gametes in *Brassica*. In addition, our study demonstrates that triploids might occasionally directly produce near-pentaploids through sexual reproduction by fusion between a reduced aneuploid gamete and an unreduced aneuploid gamete, as previously hypothesized (12). Most remarkable is our observation that allotriploid *Brassica* predominantly develops into allotetraploids with complete sets of chromosomes of *B. napus* through several generations of aneuploids.

Triploids are meiotically irregular and generate a high frequency of aneuploid gametes (9, 19). A survey of the published literature examining the cytotype composition of pollen produced by triploids found a significant positive correlation between the frequency of euploid pollen and pollen fertility, suggesting that triploids are often semifertile and that euploid gametes contribute to overall fertility (9). However, aneuploidy was reported in direct progeny of autotriploid barley (47) and *Arabidopsis* (24), as well as allotriploid *Brassica* (38). Our cytogenetic analyses show that AAC allotriploids produce predominantly aneuploid gametes at meiotic anaphase I (*SI Appendix, Fig. S4*). Our result is in agreement with the observations of ref. 24 that show that the triploid bridge acts through at least one and possibly several generations of aneuploidy.

Our results from *Brassica* strongly support the hypothesis that self-pollination plays an important role in tetraploid formation, particularly allopolyploid formation (9, 48, 49). The pathway from triploid to tetraploid by self-fertilization avoids the low rate of outcrossing involving interploidy hybridization (9), as well as

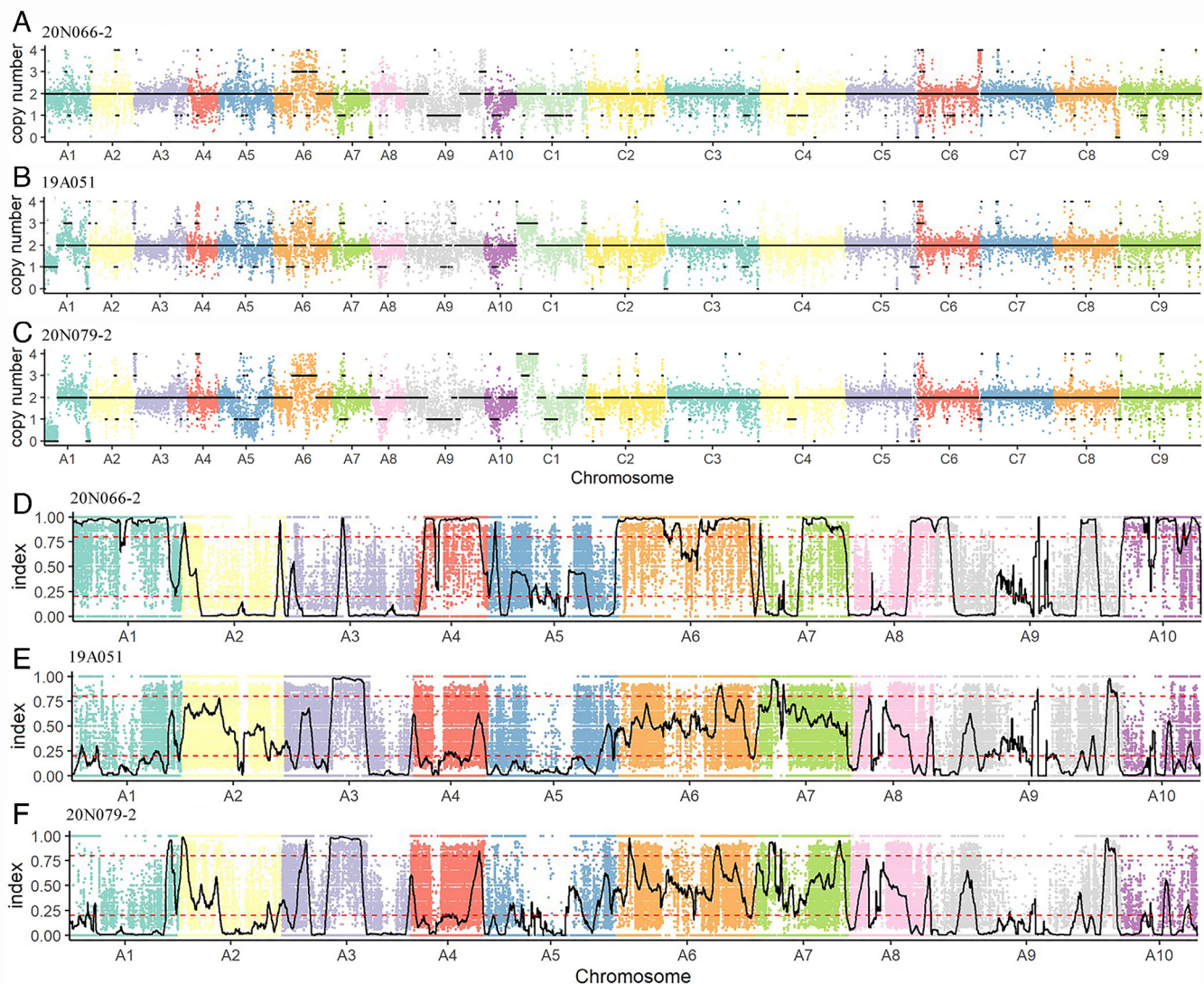


Fig. 5. Molecular karyotyping and introgression analyses of the newly formed allotetraploids among the progeny of the $A_nA_mC_n$ -M allotriploid obtained by interspecific hybridization. (A–C) Karyotyping of three new allotetraploids using bioinformatics methods. Differently colored scattered dots represent the ratio (obtained by CNV analysis) $\times 2$ (ploidy), and the black segments obtained by fitting show the copy number of each chromosome. (D–F) Introgression analysis of A–C corresponding materials. Differently colored scattered dots represent the index values of each position. The black line was obtained by sliding window analysis as described in the methods. The red dotted lines on the ordinate (index values) divide different genotypic regions. The values 0 to 0.2 and 0.8 to 1 are the homozygous regions in which the genotype of the offspring is consistent with that of their female (*B. napus* Marnoo) or male (*B. rapa* IMB218) parent, respectively, and 0.2 to 0.8 is the heterozygous region of the genotypes.

the requirement of diploid progenitors. Since allotriploid AAC *Brassica* is semifertile and the fertility of its progeny increases across generations, one allotriploid plant could produce nearly one million seeds in three generations by self-fertilization. Most of the progenies develop into allotetraploids or near-allotetraploids, which indicates that the pathway through triploid bridges via aneuploids as intermediate steps may be an efficient pathway for polyploid formation. However, more evidence from other species and natural populations is needed to confirm our proposed mechanism for the triploid to tetraploid pathway.

The Underlying Mechanism of Polyploid Formation: Genome Balance and a Dosage Effect Drive the Transition from Triploid to Tetraploid. Plants are generally more tolerant than animals to the dosage imbalance caused by aneuploidy (50, 51). As a general rule, aneuploidy is detrimental to the phenotype and organismal fertility and, in many cases, is lethal (50, 52). In addition, single chromosome addition to or subtraction from diploids has a much more detrimental effect on the organism

than whole-genome changes in both animals and plants (50, 52–54). Aneuploidy is expected to have fewer phenotypic effects in high-dosage backgrounds than those in low-dosage backgrounds (9, 55–58). In summary, the more extreme the alteration of the relative dosage of chromosomes is, the greater the observed phenotypic changes and detrimental effects (27, 35, 50, 52).

In this study, analysis of the karyotype of the immediate progeny of allotriploid *Brassica* demonstrated that all F_2 plants are aneuploids and that most of them are hypertriploids (having slightly more than the triploid number of chromosomes) containing at least two sets of A chromosomes and one complete set of C chromosomes. This skewed distribution of chromosome numbers and selection against aneuploids with fewer than 29 chromosomes in the F_2 population, which is similar to the cytometric results observed by ref. 38, suggest that increasing the genome dosage background to triploidy (or near-triploidy) results in a viable genotype. In contrast, the addition of extra C chromosomes in the dosage background of diploidy was highly detrimental and,

in most cases, lethal. Our studies also demonstrate that disrupting the genome balance of both the A and C genomes had a detrimental effect on fertility (Fig. 3 D–J).

Stoichiometric alterations of the components of certain macromolecular complexes, signal transduction pathways, or the interactome will have a negative fitness effect and be selected against (59). A and C genome imbalance induced by chromosome addition or deletion might alter the expression of hundreds of proteins, many of which belong to stoichiometric complexes (60), which may have highly detrimental effects (or be lethal), particularly to individuals with extreme alterations in genome balance.

To explain why selection is likely driving increased chromosome number toward tetraploidy, we propose that genome balance and dosage effects drive allopolyploid formation by constant selection in favor of aneuploid individuals with fewer alterations to the balance of chromosomes among the progeny of allotriploids.

Reproductive Barrier and Gene Flow in Brassica. Reproductive barriers between new polyploids and their diploid progenitors is important for polyploid establishment and persistence (22). Multiple mechanisms interact to influence the reproductive barrier between polyploids and their diploid progenitors (19, 45, 61). The interploidy hybrids between allopolyploids and diploids readily and mainly develop into abundant new allopolyploids after several generations of self-fertilization. Most remarkably, this process not only may help allopolyploids remain isolated from their diploid progenitors but also might promote allopolyploid formation, thereby facilitating allopolyploid persistence.

However, interploidy crosses also have significant effects on genetic diversity during plant evolution, enabling gene transfer between species (62, 63). The transfer of genes across a ploidy barrier has been documented between polyploids and their diploid progenitors (64–69). Unidirectional interploidy hybridization (pollen flow) has been reported in several genera (9, 26, 28), which supports the hypothesis proposed by ref. 70 that gene transfer is more likely to occur from diploid to tetraploid species, although examples of it proceeding in the reverse direction are also known (71). However, previous studies only provided results on the crossing abilities between diploids and tetraploids and the viabilities and/or fertilities of interploidy hybrids instead of supplying direct evidence that interploidy hybrids developed into true diploids or polyploids (26, 28, 64–66, 69). Evidence from *Arabidopsis* supported a role for triploids as vectors for bidirectional gene flow between diploids and autotetraploids (24). In *Brassica*, bidirectional gene flows between tetraploid *B. napus* and diploid *B. rapa* are possible via recurrent backcrossing of allotriploid hybrids to parental individuals (38, 65, 72, 73). Our study demonstrated that gene flow might be promoted from diploids to allotetraploids by the process of recurrent triploid-derived allotetraploid formation in *Brassica*, which might provide some evolutionary advantages to allopolyploids.

Materials and Methods

Plant Materials. The strategy used to produce triploids and self-fertilized progenies is shown in detail in Fig. 1. Only true triploids containing two complete sets of A genome chromosomes and one complete set of C genome chromosomes were used for cytogenetic studies and for subsequent material generation. Details of the plant materials are described in [SI Appendix, Materials and Methods and Table S1](#).

Karyotyping by FISH. The probes used for FISH, hybridization, karyotyping, and imaging were described previously (34, 36). Chromosome preparation and FISH were performed according to published protocols with slight modifications (74). Details of the experimental procedures are provided in [SI Appendix, Materials and Methods](#). The karyotypes of all the plants in different generations are provided in [Datasets S1–S3](#).

Fertility Assessment and Seed Yield. Pollen viability and seed yield were evaluated in allotriploid and self-fertilized progeny in this study. The experimental and analysis procedures are described in detail in [SI Appendix, Materials and Methods](#).

Sequencing and Data Availability. The library for Illumina sequencing was constructed and sequenced with paired-end 150 bp reads on a NovaSeq 6,000 platform (Novogene, Beijing, China). Sequencing data from this project have been deposited in the National Center for Biotechnology Information Sequence Read Archive PRJNA782082. Detailed information on the sequencing samples is shown in [Dataset S6](#).

Molecular Karyotypes and Introgression Analysis. Karyotypes of partial samples were inferred from copy number variation (CNV) analysis. Introgression analysis was similar to the QTL-seq procedure (75). The detailed bioinformatic analysis procedure is given in [SI Appendix, Materials and Methods](#).

Statistical Analysis. Statistical analyses were performed using data analysis functions in Microsoft Excel and R version v.3.6.3. Details of the statistical analysis are given in [SI Appendix, Materials and Methods](#).

Data, Materials, and Software Availability. All study data are included in the article and/or [SI Appendix](#).

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