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Meiotic stability and fertility in interspecific allotetraploid hybrids in the genus *Brassica*

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1.0 Introduction

1.1 Preface

The *Brassica* genus is the most important in the Brassicaceae family and consists of 39 species, with many of the species cultivated for their edible roots, stems, leaves, buds, flowers and seed. The six most important cultivated species of this genus and their relationship were described in U's triangle (U, 1935). They consist of three diploid species *B. rapa*, AA (2n = 2x = 20), *B. nigra*, BB (2n = 2x = 18) and *B. oleracea*, CC (2n = 2x = 18). Pairwise hybridization between these diploids gave rise to three allotetraploids *B. juncea* AABB (2n = 2x = 36), *B. carinata*, BBCC (2n = 2x = 34) and *B. napus*, AACC (2n = 2x = 38). From this, we observe that interspecific hybridization has played an important part in the evolution of species in this genus. The genetic diversity of the allotetraploids species is limited because these allotetraploid species evolved relatively recently from only a few putative hybridization events between the diploid species (Gómez-Campo and Prakash, 1999; Dixon, 2006). Therefore, interspecific hybridization offers great potential for improving the genetic diversity of these species. This will enable further genetic improvement of these species (Katche *et al*., 2019). Besides introducing new genetic diversity, interspecific hybridization can also be used to synthesize new crop types which could have significant agricultural importance (Mason and Batley, 2015).

Brassica allotetraploids can easily hybridize to produce trigenomic hybrids AABC, BBAC and CCAB (FitzJohn *et al.*, 2007; Mason *et al*., 2010). These interspecific crosses or newly formed hybrids encounter a variety of challenges, the most important of which are chromosomes pairing abnormalities and infertility (Grandont *et al*., 2014). Understanding the mechanisms behind these challenges and how to manipulate them will be important in helping

us utilize the best strategies when trying to introgress new traits and when trying to synthesize new, fertile and stable hybrids. This thesis describes the chromosome pairing behavior, inheritance, meiotic stability and fertility of *Brassica* trigenomic hybrids AABC, BBAC and CCAB formed by pairwise hybridization of *Brassica* allotetraploids in the early F_1 and S_1 generations for AABC, BBAC, and CCAB, and in the S_1 - S_6 generations for BBAC hybrids following self-pollination.

1.2 Polyploidy and interspecific hybridization in evolution and speciation

Polyploidy or whole genome duplication describes an organism or cell that contains three or more sets of chromosomes (De Storme and Geelen, 2013). Polyploidy is now recognized as an important evolutionary force not just in plants but also in animals (Leitch and Leitch, 2008; Soltis *et al*., 2015a). It is reported that 30 - 80% of all extant flowering plants are polyploids and that all angiosperms have experienced at least one round of whole genome duplication early in their evolutionary history (Jiao *et al*., 2011). Two rounds of whole genome duplication are estimated to have occurred before the divergence of extant seed plants and angiosperms, giving rise to the diversification of genes and pathways important to seed and flower development and eventually the predominance of angiosperms in the green plant clade (Song and Chen, 2015). The phylogenetic placement of these polyploidy events suggests that they might have led to key phenotypic innovations or to an increased tolerance to environmental conditions. This observation is particularly striking given that polyploidy has been postulated as an evolutionary "dead-end": additional copies of a gene mask deleterious as well as potential advantageous alleles, thus escaping selection (Schatlowski and Köhler 2012). Supporting evidence indicates that recently formed polyploids have a low diversification rate and reduced fitness, as evident in their low

pollen viability (Schatlowski and Köhler 2012.; Mayrose *et al.*, 2011). Studies using neo and synthetic polyploids have revealed that polyploidy induces distinct phenotypic and morphological changes, such as differences in flowering time and flower number, plant structure and root architecture as well as alterations in plant physiology, abiotic tolerances and other developmental process (De Storme and Mason, 2014). Some of these traits which are often absent in the diploid progenitors can enable plants to colonize new niches and to be selected for agriculture (Ramsey and Schemske, 2002; Osborn *et al*., 2003). However, the high frequency of polyploids seems to be a function of a high rate of occurrence rather than the advantages associated with recently formed polyploids (Schatlowski and Köhler 2012.). Three major advantages are often cited that should give polyploids an edge over their diploid parents. First, the increased number of alleles of a given gene following polyploidy should allow the masking of deleterious recessive mutations and thus ensure against loss of fitness (Gu *et al*., 2003). The second proposed advantage of allopolyploids and heterozygous autopolyploids is that heterosis allows offspring to display transgressive performance compared to their progenitor species (Birchler and Veitia, 2010). The third major advantage of polyploids stem from the possibility that duplicated gene copies can evolve to assume new or slightly varied functions (neo or sub functionalization) potentially allowing for ecological niche expansion or increased flexibility in organism responsiveness to environmental change (Adams and Wendel, 2005; Moore *et al*., 2005; Madlung, 2012).

There are two types of polyploids which are distinguished based mainly on the nature of the genomes present: autopolyploids which result from the multiplication of genomes derived from the same species, and allopolyploids which result from the hybridization of diverged genomes from different species (Ramsey and Schemske, 1998;

Riddle and Birchler, 2003). Some of the world's most important food crops, such as wheat (*Triticum aestivum*), rapeseed (*Brassica napus*) and cotton (*Gossypium hirsutum*) are polyploids which originated as hybrids followed by chromosome doubling (Soltis *et al.*, 2015). Hybrid speciation can occur at the same ploidy level (homoploid hybrid speciation) or more commonly though allopolyploidy (speciation via hybridization coupled with chromosome doubling). Homoploid hybrids often have greatly reduced fitness (Soltis and Soltis, 2009). Several models have been proposed to explain the origin of polyploids (Ramsey and Schemske, 1998; Chen and Ni, 2006). The two-step model proposes that allotetraploids are formed by hybridization between two species followed by chromosome doubling of the F_1 hybrids, and the one step model suggests that allotetraploids are formed by hybridization of unreduced male and female gametes from two diploid species or by direct hybridization between autotetraploids (Chen and Ni, 2006). However, the most frequent route leading to polyploid formation may be the "triploid bridge" which involves a two-step process of unreduced gamete formation and hybrids with a triploid number of chromosomes (Köhler *et al*., 2010). In the first step of the triploid bridge, an unreduced gamete fuses with a haploid gamete to produce a triploid embryo. The seeds resulting from these triploids are often non-viable (De Storme and Mason, 2014). Progeny which do successfully overcome this reproductive barrier (triploid block) encounter a problem during meiosis where the absence of chromosome pairing leads to the formation of aneuploid gametes, sterility or unbalanced chromosome sets in progeny (Ramsey and Schemske, 1998; Köhler *et al*., 2010; De Storme and Mason, 2014). However, random segregation and unreduced gamete formation in these triploids produces euploid gametes, both haploids and diploids, which may contribute to the establishment of stable polyploid population over the

course of time (De Storme and Mason, 2014).

1.3 Meiotic instability in neopolyploids and hybrids.

The establishment and maintenance of a new polyploid species is challenging. This is because numerous difficulties need to be dealt with. These include problems of meiosis leading to unbalanced chromosome numbers (aneuploidy), which can be fatal although, with a degree of fatality varying among species (Henry *et a*l., 2007). Data from molecular and phenotypic characterization of neo and synthetic allopolyploids show that the newly formed polyploids pass through a bottleneck of instabilities and fertility challenges before becoming established as new species (Comai, 2005). Meiosis is the fundamental process by which gametes of all sexual organisms are formed. Investigated for decades (Mercier *et al*., 2015; Zickler and Kleckner, 2015), this process consists of a single phase of DNA replication followed by two divisions where first, pairs of parental chromosomes (i.e., homologs) and then sister chromatids separate into four cells of a tetrad. During the first meiotic division, occurrence of meiotic recombination is critical for ensuring both genome stability and generation of diversity through crossovers. At least one crossover is required per chromosome pair to obtain well-balanced gametes and avoid formation of aneuploid progenies (Pelé *et al.*, 2018). Meiosis not only ensures fertility and genome stability but also generates diversity within species by creating new allelic combinations (Grandont *et al*., 2014; Nicolas *et al*., 2009; Gaeta and Pires, 2010).

The importance of meiosis for inheritance and evolution was first recognized more than a century ago. Since that time, considerable progress has been made in deciphering the cytological and molecular mechanisms responsible for the precise reduction

of chromosome number and the accompanying rearrangements that occur during specialized cell division (Grandont *et al.* 2013). Disomic inheritance requires that paired centromeres be aligned for equal segregation of homologous chromosomes in meiosis I and segregation of chromatids at meiosis II (Gaeta and Pires, 2010). Failure of this process results in random segregation of chromosomes, aneuploid progenies and consequently reduced fertility as observed in most newly formed polyploid plants (Grandont *et al.*, 2014). In a diploid cell, meiosis is already an intricate process in which several pathways must be coordinated to restrict recombination to homologous pairs of chromosomes. Things become even more difficult in a polyploid cell. In polyploids, the situation is delicate as it combines two genomes or more derived from the same or related species (Grandont *et al*. 2014; Stebbins, 1947; Pelé *et al.*, 2018).

In newly formed allopolyploids, meiotic recombination may also occur between the homoeologous chromosomes as reported in diverse species including *Brassica napus*, *Coffea arabica*, *Nicotiana tabacum* and *Tragopogon miscellus* (Song *et al*., 1995b; Lim *et al*., 2004; Gaeta and Pires, 2010; Xiong *et al*., 2011; Chester *et a*l., 2012). Detected as early as the first meiosis of resynthesized allotetraploids (Szadkowski *et al*., 2010), homoeologous recombination frequency often correlates with the existing collinearity between homeologs and varies according to the route of polyploid formation (Szadkowski *et al*., 2011; Rousseau-Gueutin *et al*., 2017). These homoeologous exchanges deeply impact the variability and gene content of newly formed polyploids. In *B. napus* for instance, up to 10% of genes are impacted after only three generations following resynthesis (Rousseau-Gueutin *et al*., 2017), highlighting that homoeologous exchanges are a major cause of gene copy number variation in *B. napus* varieties (Hurgobin *et al.*, 2018). In some cases, these

structural changes are the origin of phenotypic variations, such as flowering time divergence, seed quality or disease resistance (Pires *et al.,* 2004; Zhao *et al.,* 2005; Stein *et al.*, 2017), which may have contributed to the ability of allopolyploid species to exploit a wider range of environmental conditions.

Brassica napus is a model allopolyploid for the study of changes resulting from meiosis which occur in polyploid plants. *Brassica napus* is an amphidiploid species composed of homoeologous A and C genomes which are thought to have been derived from the recent progenitors of extant *B. rapa* and *B. oleracea* (Szadkowski *et al*., 2011; Cui *et al*., 2012). Natural *Brassica* allotetraploids *B. juncea, B. napus and B. carinata* show a diploid-like meiotic behavior: they are strict bivalent forming species and display an almost strict disomic inheritance. Meiotic crossovers are almost always formed between homologous chromosomes at the expense of homoeologous chromosomes, ensuring regular and stable chromosome transmission to the next generation (Liu *et a*l., 2006; Nicolas *et al.*, 2009). However, a few non- homologous exchanges (translocations) have been observed in some natural *B. napus* lines (Udall *et al.*, 2005; Parkin *et al.*, 2005; Chalhoub *et al*., 2014). Regardless, it is clear that the vast majority of crossovers during meiosis of natural euploid *Brassica* allotetraploids occur between homologous chromosomes (Nicolas *et al.*, 2009).

In contrast to extant *Brassica* polyploids which display an almost perfect disomic inheritance, newly formed *Brassica* allotetraploids encounter an immediate challenge during meiosis: homologous chromosomes must pair faithfully with each other and avoid homoeologous pairing which may lead to a breakdown of disomic inheritance resulting in complex meiotic configurations, unbalanced gametes, aneuploid progenies, chromosomal rearrangements and impaired fertility (Ramsey and Schemske, 1998). The common

evolutionary origin of the *Brassica* A, B, and C genomes which share a partially conserved genome structure and which permit homoeologous pairing to occur between them (Mason *et al.*, 2010) has been demonstrated by several studies: results show that the A and C genomes are more closely related to each other and will pair more readily than the A and B or B and C genomes (Busso *et al.* 1987; Osborn *et al.*, 2003; Liu *et al.*, 2006; Mason *et al.*, 2010). In *Brassica napus*, *Brassica juncea*, and *Brassica carinata* amphihaploids, Attia and Röbbelen, (1986) reported a high rate of pairing in *Brassica napus* (AC) amphihaploids, with an average of 12.3 bivalents per pollen mother cell (PMC) as compared to 1.2 bivalents per PMC in BC and 2.9/PMC in AB amphihaploids. In resynthesized trigenomic *Brassica* allotetraploids BBAC, CCAB, and AABC, Mason et al. (Mason *et* al., 2010) not only reported a high rate of AC bivalents compared to BC and AB bivalents but also reported autosyndentic pairing occurring between the A - A, B - B and C - C genomes.

The consequences of such pairing, i.e. the frequently observed infertility challenges encountered by newly synthesized *Brassica* allotetraploids, have been reported by several studies. In synthetic allotetraploids *B. juncea* (AABB), *B. carinata* (BBCC), and *B. napus* (AACC), obtained from reciprocal crosses of their diploid progenitors, Cui *et al*., (2012) observed that meiotic pairing was not completely diploidized, with univalents and multivalents occurring more frequently and the number of univalents found to have a negative effect on pollen viability. GISH/BAC FISH analysis revealed allosyndentic and autosyndentic pairing between the genomes with AACC genome types showing the highest rate of allosyndensis (Cui *et al*., 2012). In resynthesized *B. napus* (which is expected to have 38 chromosomes), Xiong *et al*., (2011) reported that the chromosome number varied from 2n = 36 - 42, with aneuploidy increasing in all lineages analyzed from 24.1% in $S_{0:1}$

to 71.4% in $S_{5:6}$, with up to 65% of $S_{10:11}$ lines showing aneuploidy and plants also producing fewer seeds in each successive generation. Aneuploid plants (2n > or < 38) generally had lower yield, showing the impact of incorrect meiotic pairing on fertility. Similar results have been reported in resynthesized *Brassica* allohexaploids, where most of the plants had low pollen viability and seed set as a result of the poor meiotic behavior exhibited by the hybrid plants (Tian *et al.*, 2010).

1.4 Genetic control of meiosis in polyploid plants

Crossover (CO) formation and distribution must be tightly controlled in order to maintain fertility and genomic integrity. In polyploids, there are only two allopolyploid species (wheat and canola) in which defined genetic loci have been identified that play a role in polyploid meiotic stabilization. The best-characterized is (Pairing homoeologous) *Ph1*, the locus of greatest effect in wheat. Its absence results in CO formation between homoeologous wheat chromosomes, and between wheat chromosomes and those of related species in hybrids (Sears, 1976). The *Pairing homoelogous1* (*Ph1*) locus is located on chromosome 5B where a duplication of the *ZIP4* gene within the *Ph1* locus prevents maturation of crossovers between non-homologous chromosomes (Martín *et al.* 2014; Riley and Chapman 1958; Rey *et al.*, 2017). *ZIP4* is an essential factor for the main crossover pathway (called the class I or ZMM pathway) that also includes a set of critical proteins (e.g. MER2, MSH4, MSH5, SHOC1, HEI10 and PTD) in plants (Gonzalo *et al*., 2019). Wheat lacking *Ph1* accumulates extensive rearrangements and eventually become infertile (Sánchez-Morán *et al*., 2001; Greer *et al*., 2012). Both molecular and cytological studies indicate that the absence of *Ph1* results in altered chromatin states in the early stages of meiosis (Greer *et al*., 2012), and this is correlated with increased homoeologous

chromosome pairing and recombination.

B. napus is another species in which genetic loci have been identified that regulate homoeologous recombination. Natural *B. napus* shows a predominantly diploid-like meiosis with bivalents forming at metaphase I and disomic inheritance. *B. napus* allohaploids however exhibit significant variation in meiotic behavior with most varieties displaying one of 2 meiotic phenotypes; either a high number (~10) or a low number (~4) of univalents at metaphase I (Cifuentes *et al*., 2010). Using a segregating allohaploid population derived from a cross between a low recombination and high recombination variety, numerous quantitative trait loci influencing the level of homoeologous recombination have been identified. The major determinant is *PrBn* which has been narrowed to a 10 - 20cM region on chromosome C09 (Jenczewski *et al*., 2003). In addition, 3 - 6 additive and 2 epistatic quantitative trait loci of smaller effect have also been observed (Liu *et al*., 2006). The apparent lack of variation in meiotic behavior in euploid *B. napus* indicates that *PrBn* plays a far greater role in allohaploids. In this way, *PrBn* resembles the wheat *Ph2* locus (which only shows an effect in interspecific hybrids and allohaploids) in that it only plays a significant role in the absence of homologous chromosomes. By using both cytogenetic observations and high throughput genotyping to quantify the levels of homoeologous recombination in a segregating *B. napus* mapping population, Higgins *et al*., (2021) identified three QTLs contributing to the control of homoeologous recombination in *B. napus* with one major QTL on BnaA09 contributing between 32 - 58% of the observed variation (Higgins *et al*., 2021). Five genes underlying BnaA09 were also identified including genes *RPA1C* (Replication protein A 1C) and *MUS81* (*MMS* and UV sensitive 81). It is clear that like in wheat, the regulation of

homoeologous chromosome pairing in *Brassica* is a complex trait, involving the concerted action of multiple genes (Grandont *et al*., 2014).

1.5 Genetic changes that accompany Brassica neo-hybrids and polyploids.

Stebbins 1971 concluded that multiplication of chromosome sets has little effect upon evolutionary progress at the gene level or actually retards it. However, molecular evidence suggests polyploid genomes display dynamic and pervasive changes in DNA sequence and gene expression probably as a response of "genomic shock" to genomic interactions (Chen and Ni, 2006). The evidence for genomic changes in nascent polyploid taxa comes from observation of resynthesized polyploids such as *Arabidopsis suecica*, *Brassica napus*, wheat (*Triticum aestivium)*, cotton (*Gossypium hursutum*) Triticale and *Nicotiana tabacum*; and natural polyploid taxa that have well-documented parentage such *Tragopogon*, *Senecio, Spartina* and *Glycine* (Gaeta *et al*., 2007). The genetic changes reported include deletion events (Ozkan *et al*., 2001; Shaked *et al*., 2001; Tate *et al*., 2006), gene conversion events (Kovarik *et al*., 2005), rDNA changes (Joly *et al*., 2004; Pontes *et al*., 2004), transposon activation (Madlung *et al.*, 2005), chromosomal rearrangements (Udall *et al*., 2005; Parkin *et al*., 2005; Lim *et al*., 2008; Xiong *et al*., 2011), epigenetic phenomena (Hegarty and Hiscock, 2005; Adams and Wendel, 2005; Salmon *et al*., 2005; Lukens *et al*., 2006) as well as expression level changes (Wang *et al*., 2006; Madlung, 2012).

These genetic and epigenetic changes in new allopolyploid genomes may lead to extensive gene expression changes (Chen, 2007). When two diverged genomes merge into a single cell, duplicate gene copies with similar or redundant functions may alter the expression patterns. These take several forms including unequal parental contributions,

transgressive up regulation, or down regulation, silencing and altered expression times and locations (Doyle *et al*., 2008; Yoo *et al*., 2012). The alteration of gene expression patterns is a prominent cause of the phenotypic variation between newly formed allopolyploids and their parental species and may be the primary cause of phenotypic novelty that may be selected and domesticated (Jackson and Chen, 2010; Doyle *et al*., 2018). Pires *et al.*, (2004) detected homoeologous rearrangements in resynthesized *B. napus* that altered the expression of parental *FLC* genes which are primary determinants of flowering time.

Rapid changes in genomic organization in *Brassica* synthetic allotetraploids was first reported by (Song *et al.*, 1995). They detected non-additive inheritance of genomic fragments in the synthetic allotetraploids. The changes included the absence of parental genomic fragments, and the presence of novel fragments that were absent from both parents. Many of these changes in *Brassica* allotetraploids are likely caused by reciprocal translocations as well as non-reciprocal exchanges between homoeologous chromosomes (Osborn *et al*., 2003; Zhao *et al*., 2005). Gaeta *et al*., (2007) analyzed genetic, epigenetic, gene expression and phenotypic changes in ~50 resynthesized *B. napus* lines derived by hybridizing double haploids of *B. oleracea* and *B. rapa*. Analysis of first generation S_0 found that genetic changes were rare and cytosine methylation changes were frequent. Analysis of later generations found that most S_0 methylation changes were much more frequent in S_5 generation occurring in every line. Genetic changes were detected in 36 of the 38 chromosomes of the S_5 allopolyploids and were not random across the genome.

Genome-wide gene expression changes have also been widely demonstrated in natural and synthetic allopolyploids. An initially explored issue is whether the gene expression levels observed in allopolyploids are equal to the average value of that of its

progenitor (additive) or not (non-additive). Although additive expression is prevalent, many of these expression changes are non-additive in allopolyploids where expression levels deviate from MPV (Wang *et al*., 2010; Chagué *et al*., 2010; Yoo *et al*., 2012). Wu et al., (2018) found that allopolyploid *B. napus* formation was accompanied by extensive changes (approx. one-third of the expressed genes) in the parental gene expression patterns ("transcriptome shock") with 85 of DEGs down-regulated in the allotetraploid. Approximately 36.5% of the expressed gene pairs displayed expression bias with slight preference toward the A genome. In addition to non- additive expression, homoeologue expression bias where the two homeologues are expressed unequally is commonly observed in allopolyploids, but varies among tissues and species (Flagel and Wendel, 2010; Yoo *et al*., 2012; Chalhoub *et al*., 2014; Li *et al*., 2014). Moreover, homeologue expression bias observed in parents could be maintained in the allopolyploid derivatives, indicating that the expression changes are heritable (Flagel and Wendel, 2010; Yoo *et al*., 2012; Li *et al*., 2014). Strikingly, recent findings have shown that the expression of a large proportion of genes in allopolyploids might be statistically similar to one parent but differentially expressed relative to the other (Chelaifa *et al*., 2010; Chagué *et al*., 2010; Bardil *et al*., 2011).

The merging and doubling of two genomes set in motion extensive modification of the genomes and/or transcription, with chromosomal changes such as aneuploidy creating cascades of novel expression patterns, regulatory interactions and new phenotypic variation for evaluation by selection (Adams and Wendel, 2005). In these new mergers, some duplicated gene copies lose their function, become sub-functionalized or take up new, functions (neo-functionalization). In addition, because redundancy allows gene copies to

accumulate mutation without immediate effects on the fitness of the organism, polyploidy may give rise to new allelic variants, gene family expansions and changes in gene expression (Pikaard, 2002; Roulin *et al*., 2013). The genomic shock resulting from polyploidization has also been shown to trigger transposable element activation for elements which are inactive in the progenitors as well as methylation changes, thus affecting gene activity (McClintock, 1984; Comai, 2000; Parisod *et al*., 2010).

Data suggest that a combination of genetic and epigenetic events take place quickly upon formation of a new polyploid, helping stabilize the genome and formulate coherent gene expression programs (Pikaard, 2002). Adapted polyploids that avoid extinction enter an evolutionary path of diploidization, during which genomic redundancy is reduced. Genomic changes such as DNA sequence elimination, heterochromatin expansion, reciprocal chromosome segment translocations and inversions take place, putatively helping to differentiate homologues and homeologues and ensure fertility (Comai, 2005). Duplicated genes can be lost, retained, or maintained as duplicates, undergoing sub-functionalization and neo-functionalization (Roulin *et al*., 2013). Plant evolution is now assumed to be characterized by large scale rounds of genome duplication which are then followed by selective loss of individual genes, chromosome genome fragments and associated diploidization (Gaeta and Pires, 2010; De Storme and Mason, 2014).

1.6 Interspecific hybridization in *Brassica*

Hybridization is recognized as an important process in the evolution of plants (Gross and Rieseberg, 2005; Mallet, 2007; Paun *et al*., 2009; Soltis and Soltis, 2009). Among the many effects, hybridization can result in new species of the same ploidy level (Rieseberg *et al*., 2003) or different ploidy level (Cronn and Wendel, 2004), the transfer of adaptive traits

between species (Whitney *et al*., 2010) and in general, the release of phenotypic constraints on evolution (Kalisz *et al*. 2008).

The *Brassica* genus has a history of interspecific hybridization. In this genus, interspecific hybridization between the three diploid species *B. rapa, B. nigra* and *B. oleracea* to produce the three allotetraploid species *B. juncea*, *B. napus* and *B. carinata* represents the most recent and best known polyploidization events (Zhao *et al*., 2008). To date, *Brassica* interspecific hybrids and allopolyploids have been synthesized in the lab with the aim of either introducing valuable alleles from wild relative into crop species through introgression, studying the cytological relationship between the *Brassica* A, B, and C genomes through meiotic pairing analysis, or with the aim of creating a new crop species (Abel *et al.* 2005; Meng *et al*. 1998; Zou *et al.*; Nagaharu & Nagaharu, 1935; Sundberg *et al*., 1987; Sarla & Raut, 1988; Mason *et al.*, 2012; Li *et al.*, 2014; Zhang *et al.*, 2016). Synthetic polyploids provide a model system to study the events that take place early in polyploid evolution and their consequences. Because the exact progenitor species and genotypes are known, it possible to determine the genomic changes that has occurred after resynthesis of polyploids and how this leads to speciation and evolution (Song *et al.*, 1995; Cui *et al*., 2012). Among the interspecific hybrids, *B. napus* presents an excellent opportunity for conducting cytogenetic investigation of resynthesized allopolyploids. Natural *B. napus* (2n = AACC = 38) is thought to have formed some 5000 - 10 000 years ago by hybridization between the ancestors of *B. rapa* and *B. oleracea*, which are also polyploids whose genomes are differentiated by large scale chromosome rearrangements following divergence from a common ancestor (Wang *et al*., 2011).

1.7 Usefulness of interspecific hybrids for crop improvement

Interspecific hybridization provides a means of transferring and combining desirable traits in crop species (Chen *et al*., 2011; Katche *et al*., 2019). The outcome of interspecific hybridization can be two-fold: introgression of useful alleles from one species to another, or leading to speciation. These processes can be used for genetic improvement of *Brassica* crop species (Katche *et al*., 2019). Interspecific hybridization has been used to transfer useful traits between *Brassica* species leading to significant agricultural outcomes. Genes for oil quality traits, seed color, male sterility, disease resistance and other agronomic traits of interest have all been transferred from one *Brassica* species to another. Resistance to *Leptosphaeria maculans* (blackleg) has been transferred from *B. nigra* in to the rapeseed cultivar "Darmor" (Chevre *et al.*, 1996; Yu *et al*., 2012). In a similar study B-genome chromosome was successfully transferred from *B. carinata* to *B. napus* with plants carrying this chromosome showing variation in traits such as black leg resistance (Chèvre *et al*., 1997). Other disease resistance traits which have been transferred include resistance against black rot from *B. carinata* to *B. oleracea*, resistance to leaf blight from *B. hirta* to *B. juncea* and powdery mildew resistance from *B. carinata* to *B. oleracea* (Tonguc and Griffiths, 2004; Navabi *et al*., 2010; Sharma *et al*., 2017). With regards to seed color, yellow seeded *B. napus* has been produced by interspecific hybridization of *B. napus* and *B. carinata* (Rahman *et al.*, 2001). Oil quality traits have also been successfully transferred between species. Low erucic acid and low glucosinolate content has been transferred into *B. napus* cultivars from two *B. carinata* cultivars (Friedt *et al.*, 2018). Resynthesis of *Brassica* allotetraploid species has been used to increase the genetic diversity of *Brassica* allotetraploid species. *Brassica napus* has been resynthesized by crossing *B. rapa* and *B. oleracea* to expand the existing genetic pool and to

test for new traits (Seyis *et al*., 2003; Zhang *et al*., 2004; Abel *et a*l., 2005; Girke *et al*., 2012). In the same light, *B. juncea* has been resynthesized from its progenitors *B. rapa* and *B. nigra*, with the resynthesized *B. juncea* shown to be morphologically diverse compared to natural *B. juncea* (Yadav *et al.* 2009; Prakash, 1973). *B. carinata* has also been resynthesized from its progenitor species with hybrids showing morphological variation potentially useful for crop improvement (Kirti *et al*., 1992; Jourdan and Salazar, 1993). These new synthetic polyploids serve as a source of diverse agronomic traits, where they are used to cross with and introgress new traits into high performance cultivars. All these examples go a long way to demonstrate the usefulness of *Brassica* interspecific hybrids (Prakash *et al*., 2009).

1.8 Brassica allotetraploids and genetic diversity in allotetraploids

Genetic diversity in *Brassica* allotetraploids is limited by the relatively few interspecific hybridization events in their evolution (Gómez-Campo and Prakash, 1999). *B. napus* is the most economically important of the *Brassica* crop species occupying the 3rd position worldwide in terms of the vegetable oil market. Rapeseed has been extensively bred for low erucic acid and low glucosinolate content to produce a type of rapeseed known as canola. Unfortunately, most of the genetic variation in oilseed rape has been eroded due to intensive selection for low erucic acid and low glucosinolate contents traits (Kumar *et al*., 2019). Rapeseed is not found in nature as a wild type, and most of the diversity existing nowadays comes from breeding programs or cultivars from different countries (Rahman, 2013). *Brassica juncea* is used as a vegetable, with leaf mustard or Indian mustard as the common name (Kumar *et al*., 2015). Huge diversity of leaf morphotypes is present in this species with two representative gene pools: East Europe and Indian (Banga and Banga, 2016). *Brassica*

carinata, also called Ethiopian mustard, possesses wide genetic variability and is also used as an oil seed crop (Alemayehu and Becker, 2002). It has also been considered for use in biodiesel production (Massimo Cardone *et al*., 2002) and for other purposes including as a condiment, medicine and vegetable (Kumar *et al*., 2015). Valuable genetic variation exists in various A, B and C subgenomes among the *Brassica* allotetraploid species. These species have genes for defense mechanisms against pests and disease (Roy, 1984; Chèvre *et al*., 1997; Saal and Struss, 2005). *Brassica juncea* and *B. carinata* have genes that enhance their heat and drought tolerance such as those conferring better osmotic adjustment leading to greater water use efficiency, greater radiation efficiency and deep rooting (Enjalbert *et al*., 2013).

1.9 Trigenomic hybrids and potential usefulness of new hybrids

The allotetraploid *Brassica* species *B. napus, B. juncea*, and *B. carinata* are important oilseed crop species. However, since these allotetraploids evolved relatively recently from only a few putative hybridization events between their diploid progenitors (Gómez-Campo and Prakash, 1999; Dixon, 2006), they have low genetic diversity that limits the potential for genetic improvement of cultivars. Trigenomic hybrids are hybrids with all three of the A, B and C *Brassica* genomes and provides a means to transfer genetic diversity into the oil seed forms of *Brassica* allotetraploid species (Chen *et al*., 2011).

A common approach to increase the genetic diversity in allotetraploid species is by crossing with their respective diploid progenitor species. However, this approach has two main disadvantages. First, of the three diploid progenitor species, only *B. rapa* has oilseed forms and hence crossed progenies tend to lack key oilseed agronomic characteristics such as high seed yield and high seed oil content (Dixon, 2009). Secondly, progenies of such crosses

tend to have unstable chromosome arrangements due to disruption of diploidization in the allotetraploids selected for such crosses (Song *et al.*, 1995; Szadkowski *et al*., 2010; Xiong *et al.*, 2011). As an alternative, hybrids combining the A, B and C *Brassica* sub genomes can be used to transfer genetic diversity into oilseed forms of the *Brassica* allotetraploids. In such a case, partial genome transfer may occur through the mechanism of homoeologous or homologous recombination in allotetraploid × allotetraploid hybrid combinations (Mason and Chèvre, 2016). Besides partial genome exchanges, whole genomes from different species may be substituted into agricultural cultivars using this approach, thereby increasing the genetic diversity, heterozygosity and potential yield (Chen *et al.*, 2011; Mason & Chèvre, 2016). Homologous pairing may be used if there is a very high level of similarity between the genomes of the progenitor species. Resynthesized allopolyploids have been used to introgress genetic diversity from *B. rapa* and *B. oleracea* into *B. napus* (Seyis *et al*., 2003) with successful transfer of disease resistance using this method (Rygulla *et al*., 2007). Homoeologous recombination may also occur between genomes of related species. This method has also been successfully used to transfer blackleg resistance genes between genomes in *Brassica* species (Prakash and Chopra, 1990; Chèvre *et al*., 1997; Saal *et al*., 2004).

An advantage of allotetraploid × allotetraploid crosses is that they provide the easiest means of producing hybrids containing all three *Brassica* A, B and C genomes (FitzJohn *et al*., 2007; Katche *et al*., 2019). These allotetraploid species may be crossed in different combinations to produce AABC, BBAC and CCAB hybrids which have been reported in several different experimental studies (Nelson *et al.*, 2009; Mason *et al.*, 2010; Navabi *et al.*, 2010). Through these crosses a number of agricultural improvements in *Brassica*

allotetraploids have been achieved. For example, through the use of AABC hybrids, blackleg disease resistance was transferred from *B. juncea* to *B. napus* with subsequent backcrossing to *B. napus* (Roy, 1984; Chèvre *et al.*, 1997) and from *B. carinata* to *B. napus* through CCAB hybrids (Fredua-Agyeman *et al*., 2014). Pod shatter resistance was also transferred from *B. juncea* to *B. napus* via AABC hybrids (Prakash and Chopra, 1990). The low glucosinolate canola type oil content of *B. napus* was also transferred to *B. juncea* and *B. carinata* via AABC and CCAB hybrids respectively (Getinet *et al*., 1997). Using CCAB hybrids yellow seeded *B. napus* was produced via hybridization of *B. napus* and *B. carinata* (Rashid *et al*., 1994). Resistance to White rust and Alternaria blight were transferred from *B. carinata* to *B. juncea* via BBAC hybrids (Gupta *et al*., 2010). Besides the transfer of useful genetic diversity, these hybrids can serve as models for studying meiotic stability and chromosome pairing behavior and how these affect fertility and stability of synthetic hybrids. In addition, there exists the potential for these hybrids to form new stable plants which could have important agricultural benefits. Therefore, it would be worth investigating if chromosomes in these hybrids containing three genomes can pair and recombine, leading to the recovery of stable and fertile offspring.

1.10 Aims and scope

Polyploidy and interspecific hybridization are topics which have for a long time attracted the interest of many researchers (Ramsey and Schemske, 1998, 2002; Leitch and Leitch, 2008; Soltis *et al.*, 2015a). Numerous authors have explored the cytogenetic, genetic and epigenetic changes which take place when new hybrids are formed: the immediate short-term changes such as meiotic instability, transposon activation, chromosomal rearrangements, gene expression changes and long-term changes such as gene loss, neofunctionalization, and bias fractionation which occur to further stabilize these hybrids and polyploids (Henry *et al*., 2007; Comai, 2005; Mercier *et al*., 2015). The *Brassica* genus has not been an exception. Different interspecific hybrid combinations and their resultant chromosomal, genetic and epigenetic changes have been studied and much insight has been drawn from these (Song *et al.*, 1995; Xiong *et al.*, 2011; Cui *et al.*, 2012). The allotetraploid *Brassica* species have been resynthesized and studied for the purpose of generating new hybrid crop types. However, one group of hybrids, trigenomic allotetraploids containing the *Brassica* A, B and C genomes formed by hybridizing *Brassica* allotetraploid hybrids has not been given detailed attention as to the potential of generating new stable hybrid plants. It is in this light that this thesis presents the chromosome pairing behavior, stability and fertility of *Brassica* trigenomic allotetraploids AABC, BBAC and CCAB formed by pairwise hybridization of *Brassica* allotetraploids.

The first section is a review on the importance of interspecific hybridization for *Brassica* crop improvement. In the review, we introduce the *Brassica* crop species and their wild relatives. We then discuss the barriers to interspecific and intergeneric hybridization and how to overcome these barriers before giving a summary of previous successful and unsuccessful attempts in using interspecific hybridization for the genetic improvement in *Brassica* crops.

In conclusion, we provide information about available resources to breeders who would like to take advantage of these strategies in *Brassica* crop improvement.

The first study looks at the chromosome pairing behavior, meiotic stability and fertility of AABC, BBAC and CCAB hybrids in the early F_1 and S_1 generations. For the AABC hybrids, one genotype of *B. juncea* was crossed to five different genotypes of *B. napus* to produce F_1 hybrids which were then self-pollinated. For the BBAC hybrids, one genotype of *B. juncea* was crossed with two genotypes of *B. carinata*. For CCAB hybrids, two genotypes of *B. carinata* were crossed to 12 genotypes of *B. napus* to obtain F_1 hybrid plants. The F_1 plants from these three hybrid types were then self-pollinated to produce S_1 hybrids. The fertility and chromosome pairing behavior of these hybrids were then studied.

The second study describes the meiotic stability, chromosome pairing behavior and fertility of *B. juncea* by *B. carinata* interspecific hybrids (BBAC) across six self-pollinating generations. One genotype of *B. juncea* was crossed to two genotypes of *B. carinata* to produce two hybrid lineages which were self-pollinated for six generations while assessing the chromosome pairing behavior, chromosome inheritance and fertility of these hybrids.

These hybrid combinations are peculiar and interesting because in each hybrid type, one of the subgenomes has homologous pairing partners while the other two subgenomes have no pairing partners. By using a combination of cytogenetic, molecular cytogenetic and SNP genotyping methods we wanted to study the chromosome inheritance, pairing behavior and meiotic stability of these hybrids and the effects on viability and stability. The following hypotheses were tested.

1. We hypothesized that novel stable and fertile hybrids will be recovered in later generation following self-pollination of these interspecific hybrids.
2. We hypothesized that the genome composition will affect the meiotic pairing behavior and fertility of interspecific hybrids.
3. We hypothesized that pairing and restructuring between the haploid A and C genomes in BBAC hybrids will cause them to behave as homolog.

2.0 Interspecific hybridization for *Brassica* crop improvement

2.1 Publication outline

This review paper discusses *Brassica* crop species and their wild relatives, barriers to interspecific and intergeneric hybridization and methods to overcome them. It then summarizes previous successful attempts at the use of interspecific hybridization for crop improvement in *Brassica* and provides information about resources available to breeders wishing to take advantage of this method in the *Brassica* genus.

2.2 Publication

Review

Interspecific Hybridization for *Brassica* Crop Improvement

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ABSTRACT

Interspecific hybridization is widespread in nature, where it can lead to either the production of new species or to the introgression of useful adaptive traits between species. In agricultural systems, there is also great potential to take advantage of this process for targeted crop improvement. In the *Brassica* genus, several crop species share close relationships: rapeseed (*Brassica napus*) is an ancestral hybrid between turnip (*B. rapa*) and cabbage (*B. oleracea*), and mustard species *B. juncea*, *B. carinata* and *B. nigra* share genomes in common. This close relationship, plus the abundance of wild relatives and minor crop species in the wider Brassiceae tribe which readily hybridize with the *Brassica* crop species, makes this genus an interesting example of the use of interspecific hybridization for crop improvement. In this review we introduce the *Brassica* crop species and their wild relatives, barriers to interspecific and intergeneric hybridization and methods to overcome them, summarize previous successful and unsuccessful attempts at the use of interspecific hybridization for crop improvement in *Brassica*, and provide information about resources available to breeders wishing to take advantage of this method in the *Brassica* genus.

KEYWORDS: *Brassica*; interspecific hybridization; crop improvement; crop wild relatives; genetic diversity

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INTRODUCING THE *BRASSICA* CROP SPECIES AND THEIR WILD RELATIVES

The *Brassica* genus belongs to the tribe Brassiceae (family Brassicaceae). This family comprises 338 genera (assigned to 25 tribes) and 3709 species [1,2]. The members of this family are mostly herbs with annual, biennial or perennial growth habits [3]. Initially this family was known as "Cruciferae" due to its characteristic flower conformation of four petals arranged in a cross-shape [3]. Most of the member species are

distributed in temperate regions, with the first center of diversification located in the Irano-Turranian region (~150 genera and ~900 species), followed by a second center of diversification in the Mediterranean region (>110 genera and ~630 species)[3].

Brassica is the most prominent genus in the Brassicaceae family and includes 39 species [1]. Many of the species in this genus are cultivated for their edible roots, leaves, stems, buds, flowers, mustard and oilseeds [4]. For 33 of the species the chromosome number has been determined, and ranges from $n = 7$ up to $n = 20$ [5]. During the 1930s, the chromosome number and genetic relationships between the cultivated *Brassica* species was established [6,7]. The diploid species *B. rapa* (AA, $n = 10$), *B. nigra* (BB, $n = 8$) and *B. oleracea* (CC, $n = 9$) were determined to be the progenitors of the allopolyploid species *B. juncea* (AABB, $n = 18$), *B. napus* (AACC, $n = 19$), and *B. carinata* (BBCC, $n = 17$), in a relationship known as "U's Triangle" [7]. Based on chloroplast DNA data it was determined that *B. nigra* belongs to a different lineage (Nigra lineage) than *B. rapa* and *B. oleracea* (Rapa/Oleracea lineage)[8], with the two lineages diverging approximately 7.9 Mya [9]. The divergence between *B. rapa* and *B. oleracea* has been estimated to have occurred perhaps 3.75 Mya [10] to about 5 Mya [11]. Later on, approximately 7500 years ago or less, diploid species *B. rapa* and *B. oleracea* hybridized to produce *B. napus* L. [12].

Genetic diversity within *Brassica* species has been broadly studied, with a special focus on the six crop species that form the U's triangle. Of these species, three are highly diverse: *B. oleracea*, *B. rapa* and *B. juncea* [13,14]. These species are quite morphologically variable, presenting different leaf types, numbers of branches per stem, inflorescence types, and stem thicknesses; these variations also lead to different end-product usage (e.g., oil or vegetable type)[13]. Genetic diversity observed in the *Brassica* allopolyploids can be due to (i) multiple hybridization events with diverse parents (or possibly subsequent backcrossing of the newly formed allotetraploids to the parent species) and (ii) genome changes occurring after polyploidization [15]. Four *Brassica* species are mainly used as oilseed crops: *B. juncea*, *B. rapa*, *B. carinata* and *B. napus* [16].

THE U'S TRIANGLE SPECIES AS CROPS: USES AND GENETIC DIVERSITY

Brassica napus (rapeseed, oilseed rape, swede) is the most economically important of the *Brassica* crop species, occupying the third position worldwide in the oil vegetable market, after soybean and palm oil. In the year 2016, worldwide production of rapeseed was over 68 million tons (Mt) (www.fao.org/faostat/, November 2018): In Germany, a large proportion of the rapeseed oil produced is used to generate biodiesel (2017: 4 Mt of biodiesel produced, source: European Biodiesel Board). Rapeseed, as well as other members of the Brassicaceae, naturally contain 20–40% erucic acid [17] and high glucosinolates in the seed meal.

However, rapeseed has been extensively bred for low erucic acid and low glucosinolates [18] to produce a type of rapeseed better known as canola. The main producers of rapeseed are Canada, China and India, which together represent almost 60% of the total production worldwide (www.fao.org/faostat/, November 2018). Winter-type rapeseed is mainly grown in Europe, and spring types are mostly grown in Canada, China and Australia [19]. *Brassica napus* (AACC, $2n = 4x = 38$) is thought to have originated in the last 7500 years via at least two different hybridization events between *B. oleracea* and *B. rapa* in agricultural systems [12]. Unfortunately, most of the genetic variation in oilseed rape has been reduced due to intensive selection for low erucic acid and low glucosinolate content traits [20]. Rapeseed is not found in nature as a wild type, and most of the diversity existing nowadays comes from breeding programs or cultivars from different countries [21].

Brassica juncea (AABB, $2n = 4x = 36$) is also used as a vegetable, with leaf mustard or Indian mustard as the common name [19]. A huge diversity of leaf morphotypes is present in this species that is thought to have been influenced by human selection [13], with two representative gene pools: East Europe and Indian [22]. Mustard is mainly grown in India due to climate conditions, where the breeding objectives are mainly focused on improving seed yield [16]. Although genetic resources available for *B. juncea* are not as comprehensive as those available for *B. napus* and its progenitor species, a reference *B. juncea* genome was published in the year 2016 [23].

Brassica rapa (AA, $2n = 2x = 20$), initially named *B. campestris* and commonly known as turnip or Chinese cabbage, has its origins in the Mediterranean and Central Asia [14]. The different subspecies of *B. rapa* can be used as a fodder (e.g., subsp. *rapifera*), vegetables (e.g., subsp. *chinensis* or *pekinensis*), or as an oilseed crop (e.g., subsp. *oleifera*)[14]. *Brassica rapa*, Chinese cabbage accession Chiifu-401-42, was the first *Brassica* species to get its genome sequenced [24]. Of the estimated genome size of 485 Mb, 283.8 Mb was initially assembled [24]. Later on, an improved assembly was released (v2.0) that increased the size of the genome assembly to 389.2 Mb [25]. The *B. rapa* genome is rich in transposable elements, accounting for 32.3% (~54 Mb) of the assembled sequence [25], much more than the 10.0% observed in the related genome of *Arabidopsis thaliana* [26].

Brassica oleracea (CC, $2n = 2x = 18$) is mainly used as an edible vegetable. This species is composed of several varieties and morphotypes are usually referred to as coles. These vegetables are rich in vitamin C, folate and calcium [27]. Different varieties include Brussels sprouts (var. *gemmifera*), cabbage (var. *capitata*), cauliflower (var. *botrytis*), and Chinese kale (var. *alboglabra*)[27]. In the year 2016, the worldwide production of cauliflower and broccoli surpassed 25 million tons (www.fao.org/faostat/, November 2018). Some new vegetables have also been produced by crossing different varieties within this genus, such as

broccolini [27]. Two draft genome references for *B. oleracea* were published in 2014 [28,29].

Brassica carinata (BBCC, 2*n* = 4*x* = 34), also called Ethiopian mustard, possesses wide genetic variability and is also used as an oilseed crop [30]. This crop has also been considered for use in biodiesel production [31] and for other purposes including as a condiment, medicine and vegetable [19].

Brassica nigra (BB, 2*n* = 2*x* = 16) was previously used as a condiment mustard but has now been mostly replaced by *B. juncea* [19]. Compared to the major *Brassica* crops, *B. nigra* contains little variety in physical appearance [13], but it nevertheless possesses different agronomical traits of great value such as resistance to *Phoma lingam* [32]. Although *B. nigra* is the least agriculturally significant of the six *Brassica* crop species, a scaffolded genome assembly (not yet assembled into pseudomolecules) was made available in 2016 alongside the *B. juncea* genome [23], and a new chromosome-level assembly was released in 2019 [33].

THE *BRASSICA* WILD RELATIVES: COENOSPECIES AND CYTODEMES

In the 1970s, Harberd defined the term "coenospecies" for those species or genera that have sufficient relatedness to the six *Brassica* crops to be able to exchange genetic material with them [34,35]. The coenospecies are composed of almost 100 wild species and genera that can potentially be used to increase diversity, and to introgress useful traits such as disease resistance or abiotic stress [36]. Harberd also classified the *Brassica* coenospecies into biological units called "cytodemes" [34,35,37]. Each cytodeme can contain more than one genus or species, but all species within a cytodeme should have the same chromosome number, and readily cross with other species in the same cytodeme to produce fertile, vigorous hybrids. Based on these criteria, the *Brassica* coenospecies were initially classified into 38 cytodemes [35], covering nine genera from the subtribe Brassiceae (*Brassica, Coincya, Diplotaxis, Eruca, Erucastrum, Hirschfeldia, Sinapis, Sinapidendron,* and *Trachystoma*) and two genera from subtribe Raphaninae (*Enarthrocarpus and Raphanus*). This was later updated to 63 [38], after the addition of three genera (*Moricandia*, *Pseuderucaria*, and *Rytidocarpus*) from the related subtribe Moricandiinae [39]. The crossability between cytodemes is low, but certain tools can be used to increase success rates (as discussed in later sections of this review). Crossability can also be influenced by the direction of the cross, *i.e.*, which species is used as the maternal parent, which is referred to as "unilateral incompatibility" [40]. An extended list of potentially useful agronomic traits for crop improvement present in wild allies of the *Brassica* species can be found in [41]. Examples include resistance to white rust (*Albugo candida*) in *Brassica maurorum* [42] and *Eruca versicaria* ssp. *sativa* [43], resistance to *Alternaria* blight in *Brassica fruticulosa* [44] and *Trachystoma ballii*

[45], resistance to beet cyst nematode in *Raphanus sativus* [46] and *Sinapis alba* [47], and resistance to blackleg/Phoma disease (*Leptosphaeria maculans*) in *Sinapis arvensis* [48], *Sinapis alba* [49], *Thlaspi arvense* [50], and *B. tournefortii* [51]. The *Brassica* crop species also contain unique, useful traits: examples include resistance to powdery mildew (*Hyaloperonospora parasitica*) in *Brassica oleracea* [52], resistance to clubroot disease (*Plasmodiophora brassicae*) in *B. rapa*, *B. oleracea* and *B. napus* [53], and pod shatter resistance and tolerance to heavy metals in *B. juncea* [54]. More exotic traits of interest include a C_3–C_4 intermediate photosynthetic system in *Moricandia* [55] and *Diplotaxis* species [56,57], and high erucic acid levels in *Crambe abyssinica* [58]. Cytoplasmic male sterility in *Brassica* could also be conferred by hybridization with *Sinapis incana* [59] and *Diplotaxis siifolia* [60], among other examples.

HYBRIDIZATION BETWEEN *BRASSICA* SPECIES AND WILD RELATIVES

Direct wide hybridization has been attempted many times between *Brassica* and various wild relative species, with different levels of success (reviewed in [61]). Originally such hybrids were produced to resolve chromosome homoeology (phylogenetic relationships) or simply out of curiosity [62]. However, crossing with distant relatives is today attracting increasing recognition as a method with which to improve agronomic traits in high-end varieties. There are many examples of the successful introgression of new traits into *Brassica* crops. Initial attempts to create hybrids between *Brassica* species started in the early 1800s. At this time, some crosses were made between *B. napus* × *B. rapa* and *B. oleracea* × *B. rapa*. Different success rates were reported and the results were published in 1925 by [63]. Later on, a compilation of crossability between species in the *Brassica*, *Raphanus* and *Sinapis* genera was published, showing that interspecific hybrids can be made between the *Brassica* crops and many closely-related wild species [61].

The occurrence of natural hybridization between distant relatives in natural conditions is low. For instance, [64] found that hybridization between *Brassica napus*, *B. rapa* and *B. juncea* and their two weedy relatives *B. nigra* and *Sinapis arvensis* does not occur under open pollination conditions in the field, although *B. rapa*, *B. juncea* and *B. napus* all readily produce hybrid progeny with each other under the same conditions. The cross between *B. napus* ($2n$ = 38) and *Raphanus raphanistrum* ($2n$ = 18)[65] has also been assessed under field conditions. In this case, just two allopolyploid hybrids ($2n$ = 56) were obtained from more than 52 million *B. napus* seedlings when this species was used as a female, showing a hybridization frequency of 4×10^{-8} in field conditions. These results indicate that the likelihood of this cross in the wild is low, which shows the importance of conducting such hybridizations under controlled conditions.

TRANSFER OF USEFUL TRAITS INTO *BRASSICA* CROP SPECIES THROUGH INTROGRESSION BREEDING

Disease Resistance

The introgression of genes for disease resistance between species has been widely studied in *Brassica*. One example is the utilization of the B genome as a source of resistance against *Leptosphaeria maculans* (blackleg) from diploid and tetraploid species. For instance, chromosome B4 from *B. nigra* was introgressed into rapeseed variety "Darmor", which showed high resistance with the addition of this chromosome [66]. Similarly, high resistance from *B. juncea* was obtained in selected recombinant lines of *B. napus* carrying a resistance gene located on chromosome B8 [67]. A similar study [68] successfully introgressed a B-genome chromosome from *B. carinata* to *B. napus*, with plants carrying this chromosome showing variation in traits such as blackleg resistance, days to flowering, days of maturity, and fatty acid composition. Another example is the improvement of resistance against *Erysiphe polygoni* (which can cause powdery mildew disease). Resistance in 100% of BC_1 progeny was successfully demonstrated in hybrids obtained by hand crossing and embryo rescue between *B. carinata* (donor) × *B. oleracea* [69]. Other cases of resistance transfer include transfer of blackrot resistance from *B. carinata* to *B. oleracea* [70], resistance to *Brassica* leaf blight caused by *Alternaria brassicae* from *B. hirta* to *B. juncea* [71] and transfer of powdery mildew resistance from *B. carinata* to *B. oleracea* through embryo rescue followed by backcrossing to *B. oleracea* [69].

Yellow Seededness

Yellow seededness is a desirable trait in *Brassica*, as yellow seeds have less fiber, higher protein, and higher oil content than black seeds. Although *B. juncea* and *B. rapa* contain yellow-seeded traits, this trait is not found in rapeseed (*B. napus*). Using monosomic alien addition lines from the cross *B. rapa* × *B. oleracea*, Heneen *et al*. [72] found that seven of the nine C chromosomes carry genes that affect seed color, showing the complexity of this phenotype. Interspecific crosses between *B. alboglabra*, *B. rapa* var. "yellow sarson", yellow seeded *B. carinata* and black seeded *B. napus* have been carried out previously to attempt to produce yellow-seeded *B. napus*, with interspecific hybrid progeny showing different degrees of seed colour [73]. However, this study demonstrated that the combination of the C genome of yellow-seeded *B. carinata* with the A genome of "yellow sarson" does not result in a yellow-seeded *B. napus*. The expression of this trait also appears to be heavily affected by the environment. Rashid *et al*. [74] crossed [(*B. napus* × *B. juncea*) × *B. napus*] × [(*B. napus* × *B. carinata*) × *B. napus*] and successfully obtained yellow seeds. However, when these plants were tested in the field the color was found to be highly affected by temperature [75].

Male Sterility

A common use of wild relatives for *Brassica* crop improvement is in the production of male sterile lines to facilitate hybrid production. Male sterility is often conferred when cytoplasm from an alien species is present in the genetic background of another species: this is referred to as cytoplasmic male sterility, or CMS. The most successful example of this approach in *Brassica* is the Ogura CMS system, where alien cytoplasm was obtained from crossing *Brassica napus* to Japanese radish (*Raphanus sativus*)[76]. This system was subsequently widely used in *B. napus*, *B. juncea* and *B. oleracea* [77]. Several other CMS systems have also been successfully developed from interspecific hybridization events, including a novel CMS system in *B. juncea* incorporating the cytoplasm of *B. fruticulosa* [78], and the Nsa CMS system in *B. napus* utilizing *Sinapis arvensis* cytoplasm [79]. On the other hand, several attempts to produce additional CMS lines through interspecific hybridization have also been unsuccessful. Seventeen crosses between *Diplotaxis* species and *B. napus* were done in order to introgress CMS, but out of hundreds of crossings using conventional techniques only crosses with *D. muralis* and *D. erucoides* were successful, and no CMS system was consequently established [80]. Protoplast fusion has been used to transfer *Ogu* cytoplasmic male sterility factor from *Brassica napus* to *Brassica juncea* and for the improvement of male sterile lines in hybrid breeding systems [81]. Somatic hybridization between *B. juncea* and *B. oleracea* has also been used to transfer cytoplasmic male sterility and resistance to Turnip mosaic virus from *B. oleracea* to *B. juncea* [82,83]. Prakash *et al*. [84] successfully obtained both stable CMS *B. juncea* and an introgression line carrying the restorer gene via somatic hybridization between *M. arvensis* and *B. juncea* followed by backcrossing with *B. juncea*.

Oil Quality Traits

Interestingly, oil quality traits have also been successfully transferred between species for crop improvement in *Brassica*. In the case of rapeseed, low erucic acid and low glucosinolate content originate from two *B. napus* cultivars: "Liho" with low erucic acid and "Bronowski" with low glucosinolate content [20]. Another possible source of these oil quality traits is *Capsella bursa-pastoris*, which can show less than 1% erucic acid and less than 16 μmol/g of glucosinolates in the seeds, as well as high resistance to *Sclerotinia sclerotiorum* [85]. Previously, several chromosomes and chromosomal fragments from *C. bursa-pastoris* were successfully introgressed into *B. napus* and *B. rapa* [85]. Another wild relative with favorable fatty acid content is *Orychophragmus violaceus*, which has been successfully crossed with *B. napus* [86,87]. From this cross, advanced progenies with $2n$ = 38 chromosomes, ≥70% oleic acid, 28% linoleic acid and low glucosinolate content in the seeds (<30 μmol/g oil free meal) were produced [87].

Other Traits of Agronomic Interest

Moricandia arvensis is a plant that expresses an intermediate C_3–C_4 photosynthetic mechanism [88]. This trait was introgressed into *B. napus* by somatic hybridization by [89], who obtained three hybrid plants that expressed C_3–C_4 intermediate photosynthesis characteristics. Dwarfism is a useful agronomic characteristic which helps avoid lodging, and which was introgressed by [90] from a mutant *B. rapa* into natural *B. napus* via production of a resynthesized *B. napus* from the mutant *B. rapa* with a normal *B. oleracea,* followed by four generations of backcrossing with natural *B. napus.* Pod shatter resistance has also been introgressed into *B. napus* from *B. juncea* via direct hybridization [91]. Finally, drought tolerance has been introgressed from *Sinapis alba* into *B. napus* by somatic hybridization, and was identified at the vegetative stage in the BC_3F_1 vegetation, although the original target was yellow-seededness [92].

RESYNTHESIS OF *BRASSICA* ALLOTETRAPLOID CROP SPECIES

Interspecific hybridization has two major outcomes: introgression and speciation. While introgression transfers just a limited number of alleles, hybrid speciation produces a new hybrid species. Resynthesis is the process of reproducing an already existing species from its progenitor species. This is most often done to increase the genetic diversity of the existing allotetraploid species by incorporating some of the greater genetic diversity of the progenitor species. Resynthesis as a tool of crop improvement has many benefits. Polyploidy induced during the process of resynthesis can overcome crossing barriers due to endosperm failure in interploidy crosses [93]. The genetic diversity of some *Brassica* allotetraploid crops is limited due to the few hybridization events that gave rise to these species [12]. In the case of *B. napus*, geographic isolation, extensive breeding and selection for low erucic acid and glucosinolate content has further eroded the genetic diversity of this species [37,94]. Resynthesizing the *Brassica* allotetraploids from their diploid parents is a means of increasing the genetic diversity of these species. Studies of this method abound: Seyis *et al*. [95] resynthesized 165 *Brassica napus* lines by crossing *B. rapa* and *B. oleracea* progenitor species; analysis of these resynthesized lines using RFLP markers showed they were highly genetically divergent from established oilseed rape cultivars, and also showed a high degree of morphological diversity. Abel *et al*. [96] also developed resynthesized *Brassica napus* to study fixed heterosis by crossing 21 *B. rapa* and 16 *B. oleracea* species, and showed that the direction of the cross affects hybridization outcome, although the diversity of this population and its effect on fixed heterosis was not reported in this study. Several other studies have also reported on resynthesis of *B. napus* in order to expand the available gene pool [97–100], and to test for new traits such as resistance to cabbage stem weevil *Ceutorhnchus pallidactylus* [101]. *Brassica juncea* has also

been resynthesized by crossing its progenitor species *B. rapa* and *B. nigra* to broaden the genetic base of this species [102–104]. Bansal *et al*. [105] resynthesized new *B. juncea* genotypes, and found the resynthesized *B. juncea* to be morphologically diverse compared to natural *B. juncea*. *Brassica carinata* has also been resynthesized from its progenitor species, with hybrids showing morphological variation potentially useful for crop improvement [106,107].

These new synthetic polyploids are not usually being bred to become a new crop nor in competition with the elite varieties, but rather as a source of diverse new agronomic traits, where they are used to cross with and introgress these traits into high-performance cultivars [62]. One successful example is the synthetic clubroot-resistant allotetraploid *B. napus* RS 15/04, which was created by crossing a resistant kale (*B. oleracea* ECD-15) and turnip rape (*B. rapa* ECD-04). This synthetic *B. napus* was subsequently crossed with WOSR cv. "Falcon", and a DH line created from the F_1. This line was then backcrossed with cv. "Falcon" until the BC_2F_1 where three dominant genes specific to a particular race of the clubroot pathogen were present. Further breeding was done, and in 2001 the clubroot-resistant winter oilseed rape cv. "Mendel" was released [20]. Newly synthesized *Brassica* polyploids can also present extensive genome change at very early stages and also throughout further generations (F_1–F_5)[108]. This variation can also be phenotypically observed in traits like flowering time [109] and hybrid vigor in synthetic *B. juncea* [102], and may comprise a means of generating entirely new traits.

NOVEL GENOME COMBINATIONS AND CROP TYPES

Efforts on *Brassica* improvement through polyploid synthesis have not only been limited to the naturally occurring allotetraploids. Several attempts have been made to synthesize a new, fertile and meiotically stable allohexaploid *Brassica* ($2n$ = AABBCC), with varying success rates that appear dependent on both genotype and method used (reviewed by [110]). Synthetic allohexaploids produced from crosses between *B. carinata* and *B. rapa* followed by chromosome doubling showed bigger flowers, high silique setting and high fertility, the latter increasing from the F_2 to F_4: this trend is expected to continue across generations, leading to a potentially stable species which could be of benefit to agriculture [111]. Other studies on allohexaploid *Brassica* have focused on using these hybrids as a bridge between species (reviewed by [94]), such as in the creation of novel *Brassica napus* genotypes exhibiting useful traits like yellow seededness via hybridization between *B. rapa* and *B. carinata* to produce $2n$ = AABBCC types followed by backcrossing to *B. napus* and elimination of the B genome [112–114].

The *Raphanus* genome has also been used to develop synthetic allotetraploids, as radicole (CCRR, $2n$ = 36)[115] or Raparadish (AARR, $2n$ = 38)[116]. Both of these hybrids feature a fodder-like crop

with the advantage of resistance to the beet cyst nematode. Although *B. napus* has some resistance to this nematode, transfer of this high-resistance trait from Raparadish to *B. napus* was attempted in 1993 [117]. Surprisingly, there was no significant difference in the number of hybrids produced based on the *B. napus* cultivar or accessionused in the crosses. In the F_1 population (AACR, $2n = 38$), nematode resistance was found to be intermediate between the two parental species. The meiosis observed in the F_1 plants was also very variable, producing a high frequency of unbalanced and unreduced gametes.

BARRIERS TO INTERSPECIFIC HYBRIDIZATION

Near and far relatives of major crop species provide us with an enormous untapped reservoir of agriculturally important traits. Transferring this genetic variation to crops through introgression breeding has helped produce improved, high yielding crops resilient to prevailing climatic conditions [118]. The *Brassica* A, B and C genome species and other wild relatives contain valuable genetic variation for crop improvement, including genes or alleles for defense against pests and diseases [67,69] and drought tolerance [119,120]. Extensive interspecific and intergeneric hybridization has been performed between cultivated species, and between cultivated species and wild relatives, to develop more potentially useful cultivars with improved biotic and abiotic stress tolerances [61].

However, despite the potential of using hybridization to transfer useful traits from related crop species or wild relatives, there are barriers that limit the usefulness of this process. Interspecific and intergeneric hybridization barriers can be divided into two categories: pre-fertilization and post-fertilization barriers. Pre-fertilization barrierscan arise due to failure of pollen germination, pollen tube growth or pollen tube penetration of the embryo [121,122]. Degradation or death of the hybrid embryo and male and female sterility in hybrid plants are some of the causes of post-hybridization barriers and hybrid sterility [123]. Fertilization in interspecific crosses can still occur, but later on can produce embryo abortion related to problems with endosperm development [124]. This often happens in one direction (*i.e.*, when one species is used as the maternal parent, but not when it is used as the paternal parent) and it can be overcome when the reciprocal cross direction is tested [124]. This has been recorded, and some examples show more success when *B. napus* is used as a female in interspecific hybridization events [16]. Similarly, in some attempted crosses between *B. carinata* and *B. rapa*, F_1 hybrids were only obtained when *B. carinata* was used as the female [125]. The challenge of creating interspecific hybrids increases as the phylogenetic distance between the combining species increases [126]. Opportunities for and success of interspecific crosses are also dependent on a number of other factors: physical distance between the species/parent plants, synchrony of flowering, the

specific parental genotypes used, the method of pollen dissemination, the direction of the cross (which parent is female), environmental factors, and whether one parent is male-sterile [127,128].

In *Brassica* it is difficult to make a simple statement about reproductive compatibility and incompatibility, as reproductive compatibility relationships are complicated, with partial reproductive barriers between many species [61,127]. Despite years of research on hybridization in *Brassica*, the degree of reproductive compatibility between many species combinations remains untested. Detailed summaries of the extent of interspecific hybridization in *Brassica* have been reported by various sources [13,41,61]. Given that several factors need to be considered in creating successful interspecific hybrids, different methods have been developed to transfer useful traits between different *Brassica* species and to increase the genetic diversity of *Brassica* crops.

METHODS TO FACILITATE INTERSPECIFIC HYBRIDIZATION AND THE TRANSFER OF TRAITS BETWEEN SPECIES

Early and *in Vitro* Fertilization and Embryo Rescue

Failure of foreign pollen to germinate on the stigma, to grow pollen tubes or to subsequently fertilize ovules, and for fertilized ovules to develop into seeds, are all commonly observed in interspecific hybridization attempts. However, a number of strategies exist to overcome these pre- and post-fertilization barriers (reviewed by [129]). Early pollination of stigmas (before buds open and before full maturity) or stump pollination can help in overcoming reproductive incompatibilities between some genotypes of *Brassica* species [130], while in other cases *in vitro* pollination of the stigma or pistils and/or opened ovules and ovaries may facilitate the interspecific fertilization event [131]. Seed abortion post-fertilization is also often observed in crosses between plants of different species or ploidy levels [132]. In cases where seeds cannot be obtained from crossing, a technique where the embryo is "rescued" from the putatively hostile maternal environment, usually into tissue culture or a sterile medium, can sometimes allow the production of hybrid plants. The technique of *in vitro* culture to rescue interspecific hybrid embryos was first used in crosses between *Lolium perenne* and *L. austriacum* [133]. Wide crosses between many crop plants and their wild relatives have now become possible through the use of embryo rescue techniques, as embryo rescue and subsequent culture *in vitro* helps to overcome post-fertilization barriers [70]. In the production of *Brassica* interspecific hybrids, embryo rescue is commonly used to overcome natural reproductive barriers [94,134]. Embryo rescue was first used in *Brassica* by [135]. Following this study, extensive investigations have been carried out to improve this method [136,137].

The successful application of this technique depends on the developmental stage of the embryo being rescued [70].

Several studies have demonstrated the importance and success of this technique in transferring useful traits between *Brassica* species. Using embryo rescue, triazine resistance has been transferred from *B. napus* to *B. oleracea* [133]. Yao *et al*. [138] produced allohexaploids by crossing *B. maurorum* with all three *Brassica* allotetraploids. Herbicide resistance was transferred from *Sinapis arvensis* to *B. juncea* and *B. rapa* using embryo rescue [139]. Cytoplasmic male sterility has been transferred from *B. juncea* and *B. napus* to *B. oleracea* [140]. Zhang *et al*. [98] resynthesized *B. napus* from interspecific hybridization between *B. rapa* and *B. oleracea*, and *n*ew type *B. napus* types showing resistance to *Verticillium longisporum* were synthesized from a diverse set of *B. rapa* and *B. oleracea* through embryo rescue [141].

Somatic Fusion

Somatic fusion is an important means of transferring useful traits from one species to another. Somatic fusion has the advantage that it can bypass these incompatibility barriers and transfer genes between sexually incompatible species [142]. Besides the transfer of agronomically important traits, protoplast fusion can be used to modify organellar traits, as chloroplasts and mitochondria from both parental species are combined with somatic fusion, rather than only the maternal cytoplasm being inherited by the interspecific hybrid as is the case for sexual crosses. *Brassica* species were among the first crops used for protoplast isolation, as most parts of the plant are suitable for releasing totipotent protoplasts [142,143]. Regeneration of plants from isolated protoplasts has been reported in all *Brassica* species following the first report of successful plant regeneration from *B. napus* mesophyll tissue [144]. Somatic hybridization has successfully been used to transfer traits such as disease resistance, oil quality, cold and drought tolerance and herbicide resistance between species [142,143]. In one example, somatic hybrids between *B. rapa* and *B. oleracea* were used to create improved *B. rapa* cultivars resistant to soft rot by backcrossing somatic hybrids to *B. rapa* [145]. Asymmetric somatic hybridization has also been used to transfer resistance to blackleg disease from *B. juncea*, *B. rapa* and *B. carinata* into *B. napus* [113].

Genetic Transformation

Genetic transformation can play an important role in variety improvement and functional analysis of *Brassica* crops. It has paved the way for the development of new *Brassica* varieties producing biodegradable plastics, pharmaceuticals and nutritive compounds by introducing new genes from unrelated sources [146]. Conventional breeding of *Brassica* is time consuming, labor and resource intensive. On the other hand, genetic transformation provides a direct means of

introducing specific genes or traits without negatively affecting the desirable genetic background [147]. In addition, certain important traits may not be available in the existing germplasm [148]. Under such circumstances, genetic transformation has shown to be a powerful means of effectively transferring genes across reproductive barriers [149].

Genetic transformation systems have been developed in almost all the economically important *Brassica* species, including *B. napus* [150], *B. oleracea* [151], *B. juncea* [152], *B. nigra* [153], *B. carinata*, and *B. rapa* [154]. Different plant transformation methods exist. The direct method, where naked DNA is introduced into the protoplasts of intact cells, can be mediated by methods such as polyethylene glycol (PEG) treatment, microinjection and electroporation. Alternatively, indirect methods requiring an intermediate biological vector can be used; usually *Agrobacterium tumefaciens* transformation is suitable for this purpose in *Brassica* [149].

Genetic transformation has led to the introduction of new traits in to *Brassica* crops far beyond the species boundary: genes not present in the *Brassica* species. Traits improved through genetic transformation include resistance to herbicides such as glyphosate, glufosinate, sulfonylurea, bromoxynil, and bromoxynil resistance [155–157]. Oil quality improvement has also been a target of transformation. *Brassica juncea* and *B. napus* with high oleic acid have been produced by silencing the endogenous oleate desaturase [158]. Also, transformation of the d12-desaturase genes from the fungus *Mortierella alpina* has led to the production of canola with high gamma-linolenic acid [159].

Insect and disease resistance have also been important target traits for improvement of *Brassica* crops. *Brassica napus* producing an endogenous endotoxin of *Bacillus thuringiensis* poisonous to the diamondback moth have been produced through transformation with the *Bt cry1* gene [160,161]. Novel insect resistance in *B. napus* has also been developed by transformation of chitinase and scorpion genes [161]. Transformation has been used to convert *Brassica* crops to biofactories producing pharmaceutical and industrial products such as biodegradable polymers [162]; the anticoagulant protein hirudin has been produced in *B. carinata* [163].

The development of male sterile lines and restoration system has also been a significant advancement in *Brassica* transformation. Male sterile plants were obtained in *B. juncea* by introducing the *barnase* gene with tapetum-specific promoters, following which the fertility of the male sterile line was restored by crossing it with a barstar containing transgenic line [164].

Genome Editing

Recently, the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated protein 9 (CRISPR/Cas9) system has emerged as a versatile molecular tool for genome editing in different

organisms [165]. It has been shown that the CRISPR/Cas9 system is able to achieve efficient gene editing in plants through either transient experiments or in the production of transgenic plants [166]. In this system, the endonuclease Cas9 is directed to a specific DNA target by a synthetic guide RNA [167]. It is an innovative genetic tool that can modify the genome of any species with high precision and accuracy [168]. Although this technology is still in its early stages, its application has been demonstrated not only in model species such as *Arabidopsis thaliana* [169], but also in crops such as tomato (*Solanum lycopersicum*)[170] and wheat (*Triticum aestivum*)[171]. In *Brassica napus*, proof of concept was recently demonstrated by [172], who targeted the two homologues and four alleles of the *BnALC* gene, which is responsible for fruit dehiscence in *Brassica*. Similar reports have since followed, such as [173] who determined the mutation efficiency of CRISPR-Cas9 in 12 gene families. CRISPR/Cas 9 has also been used to modify the fatty acid desaturase 2 (*FAD2*) gene which catalyzes the desaturation of oleic acid in *B. napus* leading to the production of *B. napus* with high oleic acid [168]. The application of this system has been demonstrated also in *B. oleracea* [174] and *B. carinata* [175]. CRISPR/Cas9 therefore promises to be an important tool in *Brassica* improvement. In future, linking genetic and genomic information to germplasm bank resources could extend the reach of this genome editing technique to many genetic variants of agricultural significance present within the wild relatives of the *Brassica* crop species, allowing direct editing of crops to mimic wild relative variants.

AVAILABLE GERMPLASM RESOURCES AND INFORMATION ON *BRASSICA* CROPS AND WILD RELATIVES

Wild Brassiceae species can be found around the world in temperate climates [176], and hence may constitute a valuable source of locally-adapted germplasm for use in crop improvement. Although all of the cultivated *Brassica* species are thought to originate from roughly around the Mediterranean region, with wider distributions from Europe to North Africa to the Middle East and West Asia [176], Brassiceae germplasm has also been identified in North America in archeological and ethnobotanical studies [177], with wild mustard relative *Sinapis arvensis* widespread 2000 years ago in North Eastern American states ([178] as cited in [176]). Other Brassiceae weeds and crop species have been identified in weedy habitats in Canada [179], the United States and Mexico [180,181], as well as in Australia [9], and of course Europe and Asia [176]. Germplasm resources and collections of *Brassica* crops and related species, which are either cultivated (domesticated lines) or growing in the natural environment, are mostly (90%) conserved as seeds in cold storage in gene banks [182]. These collections generally comprise elite and domesticated breeding lines, plus a few wild relatives which are being conserved for breeding as well as for research purposes. Overall,

conservation methods can basically be categorized into either *in situ* or *ex situ* conservation.

In Situ Conservation of Germplasm Resources

In situ conservation is the primary form of conservation for crop wild relatives, and either takes place in farmers' fields or in natural environments. *In situ* conservation is promoted because landraces can be an essential component of indigenous cultures and show highly specialized local adaptations [183,184]. Growth of plants in the natural environment also allows selection and adaptation to changing environmental conditions and is highly cost effective [182]. Growing interest in the use of wild species in breeding [185,186] has underlined the need to also create national *in situ* inventories to encourage conservation. *In situ* conservation also includes conservation in natural or wilderness areas, national parks and special management areas. Understanding the genetic potential of *Brassica* crops and wild relatives is critical for the establishment of long term breeding programs. Useful agronomic traits which can potentially be introgressed from wild relatives into elite crops include resistance traits [176], salt tolerance [187,188] and cold tolerance [189]. However, to date *ex situ* conservation remains the most common form of germplasm conservation.

Ex Situ Conservation of Germplasm Resources: Genebanks

Ex situ conservation of plant genetic resources started in themid-twentieth century, as an initiative to prevent the rapid loss of plant biodiversity resulting from the introduction of improved varieties to replace landraces [182,190,191]. Therefore, germplasm (or "gene") banks were established with the intention to preserve genetic material which might be useful in future for cultivation or as material in breeding programs [192]. The major world germplasm collections of *Brassica* today include the Centre for Genetic Resources (CGN, The Netherlands), the Institute for Horticultural Plant Breeding (IVT, The Netherlands), the Horticultural Research Institute (HRI, UK) and the Gene Bank of the Crop Research Institute (UK)[193]. Other genebanks include the United States Department of Agriculture (USDA) (https://www.ars-grin.gov/npgs/) in the United States, the Australian Grains Genebank (https://grdc.com.au/resources-and-publications/groundcover/gc110/australian-genebank), and the Nordic Genetic Resource Centre (NordGen) (https://www.nordgen.org/en/) in Norway. In Spain, the *Brassica* genebank MBG-CSIC (http://www.mbg.csic.es/es/) started its activities in 1985. This gene bank holds a collection of Galician *Brassica* crops belonging to the species *B. oleracea* L., *B. rapa* L. and *B. napus* L., and houses a total of 644 accessions. *B. oleracea* varieties include kales (*B. oleracea* var. *acephala*), cabbages (*B. oleracea* var. *capitata*), and Tronchuda cabbage (*B. oleracea* var. *costata*). *Brassica rapa* includes the turnips, turnip greens, and turnip tops; and *B. napus* appears only in the

form known as "nabicol" or leaf rape [194]. The United Kingdom Vegetable Genebank (UKVGB) managed by the University of Warwick conserves approximately 14,000 accessions of crops including *Brassica* types [195]. *Brassica* genetic resources hosted at the UKVGB have been incorporated into several germplasm panels, including (amongst others) the European clubroot differential series (ECD) to help identify races of the clubroot-causing pathogen *Plasmodiophora brassicae* [196], *Brassica S* allele (self-incompatibility) collections [195] which comprise *Brassica* lines with characterized *S*-allele haplotypes, and other collections of *B. oleracea* and *B. napus* fixed diversity sets (homozygous doubled-haploid (DH) or inbred lines)[197]. In total, about 74,000 *Brassica* accessions from various sources have been identified: mostly conserved in Europe (41%) and Asia (41%) as well as a few in the Americas (12%)[38]. *Brassica oleracea* and *B. rapa* species, which comprise the most important *Brassica* vegetables, are represented worldwide by about 20,000 (27%) and 18,000 (25%) accessions, respectively [198]. The European *Brassica* database (Bras-EDB; www.cgn.wageningen-ur.nl/pgr/collections/brasedb/) contains detailed accession data on 32 collections from 22 European countries.

A total of 412 accessions of wild relatives have also been identified in gene banks (mostly European) including 179 species at the University of Madrid in Spain, and 97 species at the Leibniz-Institut für Pflanzengenetik und Kultur Pflanzenforschung (IPK) in Gatersleben, Germany [199]. However, wild species are still under-represented in most *ex situ* collections [198].

Information Databases

Brassica databases are another important resource for crop improvement. These comprise freely available online databases which provide genomic and genetic data for important *Brassica* crops, including genome sequence information, predicted genes and associated annotations, and genetic marker information. In addition, several databases provide cytogenetic and taxonomy data, such as Brassibase (https://brassibase.cos.uni-heidelberg.de/), or species distribution and observation data (usually for specific countries or regions) for *Brassica* crops and wild relatives growing in the natural environment. In Canada for example, an electronic database provides taxonomy and synonymy information for 338 Brassicaceae genera and 3709 species (14,000 taxonomic names) found distributed across Canada: http://www.cbif.gc.ca/eng/species-bank/?id=1370403266204 [1]. The *Brassica* database (BRAD (https://brassicadb.org)) has a specific focus on genome annotations and deep mining of the assembled *Brassica* crop genomes to provide information for breeding and research [200]. Another database, brassica.info, contains links to browsers and downloads for annotated reference genomes of *B. napus*, *B. rapa* and *B. oleracea* as well as *Brassica* linkage maps and molecular marker collections (www.brassica.info/

genome/linkage_maps.html). The *Brassica* genome databases (http://www.plantgdb.org/BrGDB) mainly focus on genome data dissemination via CropStore and the *Brassica* Genome Database (*Brassica*DB). The *Brassica* CropStore was initially developed to collate and disseminate information from crop research communities [201,202] as well as provide data information for *Brassica* phenotypic and genetic maps from different projects [203,204]. CropStore is an integral part of InterStoreDb which provides a platform for the utilization of a set of interlinked databases to assist linking phenotype to QTL regions for a particular trait. Data contained within CropStore can be accessed via a web interface [201,204].

In the era of fast growing technologies such as genome editing, sequencing and biotechnology tools, there is scope to improve the efficient utilization of information and resources provided by gene banks. Future gene banks should also aim to conserve DNA as well as products of genome editing and transgenic approaches, alongside genomic sequence information for plant accessions [194]. If possible, current gene banks should aim to provide genotypic as well as phenotypic information on *Brassica* species and wild relative collections in the form of an online portal or databases. A number of online *Brassica* species databases have been in existence since the era of reduced cost genome sequencing: the incorporation of these online databases with traditional germplasm banks would provide breeders and scientists with considerable resources for efficient crop improvement.

FUTURE OUTLOOK

In this review we describe the progress that has been made to date in the use of interspecific hybridization for *Brassica* crop improvement. But what may be possible in future? Recent technological advances in genome sequencing and editing have the potential to revolutionize the use of genetic diversity present in the wild relatives for *Brassica* crop improvement. Putatively, *Brassica* wild relatives with useful phenotypic diversity can be identified through screening of diverse populations under different environmental conditions, phenotype data then coupled with genome and resequencing data to link phenotypes to genotypes, followed by gene editing to directly install these genetic variants into the major *Brassica* crop species. Although this process may still be more speculative than realistic, the technological basis for this approach already exists today. High-throughput phenotyping platforms are available and under constant improvement for glasshouse and field environments [205–207]. In natural environments, traits have also been successfully linked to genetic loci through sequencing of contrasting species populations in different habitats [208]. Whole genome sequencing and resequencing is becoming increasingly cheap and available, with major strides being made in both improving genomic resources available for the *Brassica* crop genomes [209–212] and in the availability of

additional genomic resources for *Brassica* wild relatives [213,214]. As previously mentioned, genetic transformation and genome editing protocols have already been established for many of the *Brassica* crop species [172,174,175]. In future, we expect the true value of interspecific hybridization and the use of wild relatives for crop improvement in the agriculturally significant *Brassica* genus to be realized, with implementation of new technologies supported by gene banks and information resources for breeding and research outcomes.

AUTHOR CONTRIBUTIONS

All authors contributed to conceptualization and writing of the review.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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3.0 Genome composition in *Brassica* interspecific hybrids affects chromosome inheritance and viability of progeny

3.1 Publication outline

The following publication describes the fertility and chromosome inheritance of trigenomic *Brassica* interspecific hybrids of AABC, BBAC and CCAB formed by pairwise hybridization of the *Brassica* allotetraploid species in the F_1 and their self-pollinated progeny. Fertility was generally low in these hybrids though BBAC hybrids had a higher seed fertility compared to AABC and CCAB. A strong bias towards retention than loss of haploid genomes was observed. Our results suggest that the relationship between subgenomes determine hybridization outcomes.

3.2 Publication

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RESEARCH

Genome composition in *Brassica* interspecific hybrids affects chromosome inheritance and viability of progeny

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Abstract Interspecific hybridization is widespread in nature and can result in the formation of new hybrid species as well as the transfer of traits between species. However, the fate of newly formed hybrid lineages is relatively understudied. We undertook pairwise crossing between multiple genotypes of three *Brassica* allotetraploid species *Brassica juncea* (2*n* = AABB), *Brassica carinata* (2*n* = BBCC), and *Brassica napus* (2*n* = AACC) to generate AABC, BBAC, and CCAB interspecific hybrids and investigated chromosome inheritance and fertility in these hybrids and their self-pollinated progeny. Surprisingly, despite the presence of a complete diploid genome in all hybrids, hybrid fertility was very low. AABC and BBAC first generation (F_1) hybrids both averaged ~16% pollen viability compared to 3.5% in CCAB hybrids: most CCAB hybrid flowers were male-sterile. AABC and CCAB F_1 hybrid plants averaged 5.5 and 0.5 seeds per plant, respectively, and BBAC F_1 hybrids ~56 seeds/plant. In the second generation (S_1), all confirmed self-pollinated progeny resulting from CCAB hybrids were sterile, producing no self-pollinated seeds. Three AABC S_1 hybrids putatively resulting from unreduced gametes produced 3, 14, and 182 seeds each, while other AABC S_1 hybrids averaged 1.5 seeds/plant (0–8). BBAC S_1 hybrids averaged 44 seeds/plant (range 0–403). We also observed strong bias towards retention rather than loss of the haploid genomes, suggesting that the subgenomes in the *Brassica* allotetraploids are already highly interdependent, such that loss of one subgenome is detrimental to fertility and viability. Our results suggest that relationships

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between subgenomes determine hybridization outcomes in these species.

Keywords *Brassica* · interspecific hybridization · homoeologous exchange · hybrid stability · hybrid fertility · introgression

Introduction

The *Brassica* genus is the most prominent in the Brassicaceae family and includes 39 species. Many of the species in this genus are cultivated for their edible roots, stems, leaves, buds, flowers, and seeds (oil and mustard) (Rakow 2004). The six most agriculturally important members of this group are described by the Triangle of U, which also established the chromosome number and genetic relationship between these cultivated species. The Triangle of U consists of three diploid and three allopolyploid species: diploid species *Brassica rapa* (*B. rapa*) (A genome, $n = 10$), *Brassica nigra* (*B. nigra*) (B genome, $n = 8$), and *Brassica oleracea* (*B. oleracea*) (C genome, $n = 9$) were determined to be the progenitors of the allopolyploid species *Brassica juncea* (*B. juncea*) (AB genomes, $n = 18$), *Brassica napus* (*B. napus*) (AC genomes, $n = 19$), and *Brassica carinata* (*B. carinata*) (BC genomes, $n = 17$) (U N 1935).

The ancestral relationship which exists between the *Brassica* A, B, and C genomes has been well elucidated (Attia and Röbbelen 1986; Lagercrantz and Lydiate 1996; Ge and Li 2007; Mason et al. 2010; Chalhoub et al. 2014). The A and C genomes have been shown to be more closely related to each other than to the B genome, with the *B. nigra* (B) lineage predicted to have diverged from the *B. rapa* and *B. oleracea* (A/C) lineage approximately 7.9 million years ago (Mya) followed by the separation of the *B. rapa* (A) and *B. oleracea* (C) lineages about 3.7 Mya (Inaba and Nishio 2002; Panjabi et al. 2008). Studies have also shown that the A and C genomes readily pair with each other. This has been demonstrated in synthetic AACC allotetraploids (Katche and Mason 2023), AC haploids (Nicolas et al. 2009), AAC and CCA triploids (Leflon et al. 2006), in trigenomic AABC, BBAC, and CCAB tetraploid hybrids (Mason et al. 2010), and in AABBCC allohexaploids (Gaebelein et al. 2019a, 2019b), with the highest frequencies observed in the absence of homologous chromosome pairing partners in the A and C genomes (allohaploids)(Nicolas et al. 2009). Although the B genome still shares a high degree of homoeology with the A and C genomes (Lagercrantz and Lydiate 1996; Perumal et al. 2020), A-B and B-C homoeologous pairing is less frequently observed (Mason et al. 2010; Chen et al. 2011; Navabi et al. 2011; Gaebelein and Mason 2018). The diploid A, B, and C genomes are also mesopolyploid, with a triplicated structure resulting from ancestral polyploidy events in the Brassiceae lineage (The Brassica rapa Genome Sequencing Project Consortium 2011; Parkin et al. 2014). These regions of secondary homoeology are also sufficient to induce chromosome pairing (autosyndesis) at low frequencies, e.g., in A genome (Armstrong and Keller 1981), B genome (Prakash 1973), and C genome (Armstrong and Keller 1982) haploids, and in AABC, BBAC and CCAB hybrids (Mason et al. 2010).

Allotetraploid × allotetraploid *Brassica* crosses can be readily carried out to produce hybrids containing all three *Brassica* A, B, and C genomes (FitzJohn et al. 2007; Katche et al. 2019). These allotetraploid species may be crossed in different combinations to produce AABC, BBAC, and CCAB hybrids which have been reported in several different experimental studies (Nelson et al. 2009; Mason et al. 2010; Navabi et al. 2010). Recently, we reported the fate of BBAC hybrids (Katche et al. 2021), but to date, very little is known about how AABC and CCAB hybrid lineages behave in subsequent generations following self-pollination. Each of the three allotetraploid parent species is self-compatible, so self-pollination success is expected to be a product primarily of the meiotic process and chromosome inheritance in these hybrids. In this study, we describe the production of three *Brassica* interspecific hybrid types; AABC, BBAC, and CCAB, by pairwise crossing of different self-compatible genotypes of the *Brassica* allotetraploid species *B. juncea*, *B. napus*, and *B. carinata*. We studied the chromosome inheritance, fertility, and stability of these hybrids following self-pollination, in order to shed light on possible pathways for natural hybridization and species formation.

Materials and methods

Experimental plant material and growth conditions

Three *Brassica* trigenomic hybrid populations AABC, BBAC and CCAB obtained by interspecific hybridization of *Brassica* allopolyploids were used for this study. Fertility for a subset of first generation (F_1) hybrid plants and genotypes used in this study is presented in Mason et al. (2011). BBAC F_1 and second generation (S_1) hybrid types (derived from self-pollination of the F_1 hybrids), along with information on the later generations of this cross, have already been described in detail (Katche et al. 2021) but are presented again here for the purposes of comparison between the three hybrid types. All AABC S_1 and CCAB S_1 data are newly presented, including fertility data (Supplementary File S1b-g), chromosome counts (Supplementary File S1f-g), and SNP genotyping information (Supplementary File S2a, b).

Brassica juncea genotype "JN9-04", hereafter represented with the code "J1", was crossed with five different genotypes of *Brassica napus* (Boomer, Monty_028DH, Surpass400_024DH, Trilogy, and Westar_010DH) from Canola Breeders Western Australia to obtain 133 AABC F_1 hybrids. A total of 93 AABC S_1 plants were produced by self-pollination of AABC F_1 plants. One hundred and twenty four CCAB F_1 plants from ten different cross combinations were obtained by crossing two genotypes of *B. carinata* (195923.3.2_01DH and 94024.2_02DH) hereafter referred to as "C1" and "C2", with twelve genotypes of *B. napus* (Ag-Spectrum, Argyle, ATR Cobbler, AV-Sapphire, and Skipton from the Australian Grains Genebank (Mason et al. 2015); Ningyou7 from Huazhong Agricultural University; and Boomer, Surpass400_024DH, Monty_028DH, Trilogy, Westar_10DH, and Lynx_037DH from Canola Breeders Western Australia (Supplementary File S1a). Sixteen CCAB S_1 plants were subsequently produced by self-pollination of CCAB F_1 plants derived from crosses between the two aforementioned genotypes of *B. carinata* and three genotypes of B. *napus* (Surpass400_024DH, Boomer, and Trilogy). The parental *B. juncea* genotype J1 was crossed with two different *B. carinata* genotypes "C1" and "C2", respectively, to generate two separate BBAC F_1 hybrid genotypes: "J1C1" and "J1C2." A total of sixty-two BBAC F_1 plants were produced. Two hundred and twenty-seven BBAC S_1 seeds produced from self-pollination of the J1C1 and J1C2 BBAC F_1 genotypes were sown directly into the field at Huazhong Agricultural University, Wuhan, China. An additional 44 seeds were grown under glasshouse conditions at The University of Queensland, while seeds from all other BBAC F_1 hybrids were germinated in potting mix and grown in pots in a controlled environment room (CER) at 18 °C/13 °C day/night with a 16 h photoperiod and light intensity of approximately 500 µmol m-2s-1. The three most fertile plants from each BBAC F_1 plant, as measured by total self-pollinated seed produced, were selected to produce the next generation (one parent was selected from the growth room condition, five from the field condition).

Fertility data collection

Total seed set was counted for all plants after encouraging self-pollination using micro-perforated plastic sleeves or paper bags to enclose racemes. Newly opened flowers were collected when plants started flowering and pollen stained with either fluorescein diacetate using the method detailed by Heslop-Harrison et al. (1984) (for the plants described in Mason et al. 2011, only pollen which fluoresced bright green were assumed to be viable) or with 1% acetic acid carmine stain (all other plants, plump and darkly stained pollen were assumed to be viable). At least 300 pollen grains were counted for each of two flowers per plant and the percentage pollen viability was recorded (Supplementary Files S1b-g). Plants were then bagged to encourage self-fertilization, and total seed counted after drying (Supplementary Files S1b-g).

Plant hybrid status

As all parent species and genotypes in the cross combinations were self-compatible, several measures were taken to establish true hybrid status for progeny resulting from interspecific hybridization (Supplementary File S1b-g). For first generation hybrids (F_1), plants were scored on one or multiple of the following: (1) plant morphology, (2) pollen morphology, (3) microsatellite marker inheritance of single alleles from both parents (see Mason et al. 2011 for details), or (4) whole-genome SNP array genotyping for parent allele inheritance. Plant morphological traits scored

included leaf, stem and flower color, leaf margin serration, leaf lobe number and morphology, leaf and stem hairiness, and growth habit. The majority of F_1 progeny sets resulting from specific parent combinations could be definitively characterized based on these phenotypic traits, with confirmation provided from microsatellite marker results (see Mason et al. 2011). Hybrid pollen morphology was also distinctive: pollen from true hybrid plants showed large size variation between pollen grains and more spherical appearance (as opposed to ovoid) for viable pollen in comparison to the pollen of the parent species (see Mason et al. 2011 for details). For second-generation progeny resulting from self-pollination of true F_1 hybrid plants (S_1 generation), only genome-wide SNP genotyping to determine if no unexpected alleles were present relative to the alleles predicted from the two parents was considered sufficient to distinguish between truly self-pollinated progeny and progeny resulting from foreign pollen contamination on to the maternal F_1 plants.

DNA extraction and marker-based genotyping for the AABC, BBAC, and CCAB hybrids

Leaf samples were collected in 2-ml micro-centrifuge tubes and stored at −20 °C until use. DNA was extracted using the "Microprep" method described in Fulton et al. (1995), except for 30 AABC plants which were extracted using the BioSprint 96 plant work station (Qiagen, Hilden, Germany). Sixty-one AABC hybrids, forty BBAC, and eighteen CCAB S_1 hybrids were genotyped. All BBAC and CCAB hybrids and 31 AABC hybrids were genotyped using the Illumina Infinium 60K *Brassica* AC SNP array (Clarke et al. 2016). The remaining 30 AABC hybrid plants were genotyped using the Illumina Infinium 90K *Brassica* ABC SNP array. Hybridization protocols were performed according to the manufacturer's instructions for all samples and the genotype data was visualized and exported using the Genome Studio v2.0.4 software (Illumina Inc., San Diego CA, USA). A total of 52 149 SNPs were exported for the A and C genomes (Supplementary File S2a,b). Through BLAST alignment of the SNP probe sequences, A- and C-genome SNPs were located on the Damor-*bzh* v8 reference sequence (Bayer et al. 2017). SNP genotyping analysis followed established methodology (Mason et al. 2017). Briefly, for downstream analyses, SNPs which had a "no call" in > 10% of individuals within a haplotype block ($r^2 = 1$) of called SNPs or which had a "call" in > 10% of individuals within a haplotype block ($r^2 = 1$) of "no-call" SNPs were removed from all hybrid types, in addition to SNPs showing patterns of segregation inconsistent with determined genomic locations. For the AABC S_1 hybrids, the A genome was filtered to retain only SNPs which were polymorphic between the parent *B. napus* and *B. juncea* genotypes in the A genome, while in the CCAB S_1 hybrids, the C genome was filtered to retain only SNPs which were polymorphic between the parent *B. napus* and *B. carinata* genotypes for each hybrid combination. No allelic segregation was expected for the B and C genomes in AABC hybrids, the A and C genomes in BBAC hybrids, or for the A and B genomes in CCAB hybrids, and hence SNPs which were heterozygous within these genomes (indicative of multi-locus amplification/aspecific probe binding) were filtered out with respect to parental genotype controls. As well, SNPs which mapped to the A genome but which amplified in *B. carinata* ($2n$ = BBCC) and SNPs which mapped to the C genome but which amplified in *B. juncea* ($2n$ = AABB) were filtered out. S_1 generation individuals for each of the AABC, BBAC, and CCAB hybrids were determined to be the product of unintentional cross-pollination when these individuals showed presence of alleles (in haplotype blocks, not individual SNPs which might result from errors) that were not present in either of the two parent genotypes.

Molecular karyotyping

Molecular karyotyping was carried out in order to establish the number of chromosomes present in each of the A and C genomes (and B genome if genotyped) and the presence of non-homologous recombination events. Centromere locations used were initially mapped using the Darmor v.4.1 *B. napus* reference genome using the half-tetrad analysis (see Mason et al. (2016) for details) and subsequently relocated on the Darmor v. 8. 1 *B. napus* reference genome ((Bayer et al. 2017); see Katche et al. (2021) for reported positions). Presence of a centromere was taken as evidence for presence of a chromosome, regardless of other putative non-homologous translocation events present on that chromosome, as chromosome fragments without a centromere cannot be transmitted

via mitosis or meiosis. In chromosome regions spanning at least 10 SNPs and > 1 Mbp, strings of no calls (NC) in the genotyping data were taken as indications of absence of this chromosome region, indicative of a non-homologous recombination event (Mason et al. 2017; Quezada-Martinez et al. 2022).

Cytological chromosome counting

Root tips were collected in 0.04% 8-hydroxyquinoline solution and incubated for 2 h at room temperature, followed by another 2 h at 4 °C. Root tips were then transferred to Carnoy's I solution (3:1 parts ethanol: acetic acid) and incubated for 24 h before being transferred to 70% ethanol for storage at −20 °C. The procedure for mitosis slide preparation from root tips was as reported by Mason et al. (2014a), using the DAPI as the fluorescent stain. Fluorescence images were captured using a Cool Snap HQ camera (Photometrics) on an Axioplan 2 microscope (Zeiss) and analyzed using the MetaVue (Universal Imaging).

Statistical analysis and graphing

Genotypic effect of the trigenomic hybrids on total number of self-pollinated seeds and pollen viability was tested for using the one-way ANOVA in the base R version 4.0.2 (R_Core_Team 2022), followed by Tukey's Honest Significant Differences test for post-hoc comparisons between hybrid types. The one-way ANOVA was also used to test for significant differences in genotype for the total number of self-pollinated seeds in the F_1 and S_1 generation of AABC, CCAB, and BBAC hybrids. Boxplots and stripcharts were also produced in the base package of R v.4.0.2. Pearson's χ^2 test statistic values and barcharts were produced using the Microsoft Office Excel (2019), and chromosome images were combined and annotated using the Microsoft Office Powerpoint (2019).

Results

True hybridity (F_1) and self-pollinated (S_1) status of AABC, CCAB, and BBAC hybrid plants

A total of 125 plants were obtained from five different cross combinations of *B. juncea* and *B. napus* (Supplementary File S1b). Of these, 123 plants were predicted to be true F_1 hybrids (based on plant morphology, pollen morphology, and/or marker data), while two plants were derived from maternal self-pollination (Supplementary File S1b). Of the 123 F_1 hybrid plants, two were found to derived from unreduced gametes produced by the maternal (*B. napus*) parent and hence to have a genome complement of $2n$ = AAABCC; these showed morphology similar to the maternal parent (Mason et al. 2011). The remaining hybrids were assumed to have $2n$ = AABC chromosome complements. To produce the BBAC hybrids, one genotype of *B. juncea* was crossed with two genotypes of *B. carinata*. A total of 62 plants were obtained, all of which were true F_1 hybrids; one of these plants was phenotypically abnormal (and sterile) and was found to derive from an aneuploid (< n) *B. carinata* gamete (Mason et al. 2011) (Supplementary File S1c). To produce the CCAB hybrids, two genotypes of *B. carinata* and 12 different genotypes of *B. napus* were hybridized to produce 121 plants. Of these, 116 plants were true F_1 hybrids and five plants were derived from self-pollination of the maternal parent (Supplementary File S1d).

AABC, CCAB, and BBAC F_1 hybrid plants were self-pollinated in order to obtain S_1 hybrid plants. A total of 93 AABC S_1 hybrid plants were produced by self-pollinating F_1 hybrids, with SNP genotyping data available for 78 S_1 plants (Supplementary File S1e). Of the SNP-genotyped plants, 10 plants (13%) were true S_1 generation resulting from self-pollination, and 68 plants (87%) resulted from unintentional cross-pollination. The status of the 15 plants which were not SNP-genotyped could not be determined. A total of 191 BBAC S_1 plants were produced. From these, SNP genotyping information was only available for 38 plants. All 38 plants were true S_1 self-pollinated progeny of their F_1 hybrid parent based on SNP information (Supplementary File S1f; (Katche et al. 2021)). For the CCAB plants, 15 S_1 hybrids were obtained, out of which 11 plants were SNP genotyped. Five of these 11 plants were true self-pollinated progeny, while six plants resulted from unintentional cross-pollination (Supplementary File S1g).

Fertility of AABC, CCAB, and BBAC interspecific F_1 hybrids

Fertility was assessed using seed set and estimated pollen viability as fertility measures in all true AABC, CCAB, and BBAC F_1 hybrids (Supplementary File

S1b-d). Pollen viability data was collected for 61 out of 121 AABC F_1 lines and ranged from 2 to 51%, with an average of 16.6% (Fig. 1). The genotype combination *B. juncea* (JN9-04) × *B. napus* (Boomer) had the highest pollen viability while *B. juncea* (JN9-04) × *B. napus* (Monty_28DH) had the lowest. Bagged seed set was obtained for 91 out of 121 AABC F_1 plants and ranged from 0 to 176 seeds per plant (Fig. 2), with an average seed fertility of 5.5 seeds per plant. Nearly half (43%) of the plants did not set any seed when bagged, and no significant difference between genotypes of different cross combinations for bagged seed set was observed ($p < 0.05$).

For the CCAB hybrids, a total of 116 F_1 hybrid plants were produced. Pollen viability data was obtained for 73 true F_1 plants and ranged from 0 to 29%, with an average of 3.5% (Fig. 1). Of all hybrid types, CCAB hybrids had the lowest percentage of viable pollen, with no viable pollen produced in 37% of plants. Number of seeds produced after bagging was obtained for 76 out of 116 hybrid plants, ranging from 0 to 9 seeds per plant, with an average of 0.5 seeds per plant (Fig. 2). Most (87%) of these plants did not produce any seed (Fig. 2), and no significant difference was detected between genotypes ($p < 0.05$).

For the BBAC hybrid population, a total of 62 true F_1 hybrid plants were produced. Pollen viability data was obtained for 31 of these plants, which ranged from 0 to 59%, with an average of 16% (Fig. 1**)**. There was no significant difference between the two hybrid combinations J1C1 and J1C2 in terms of pollen viability (ANOVA, $p = 0.164$). Seed data in BBAC hybrids was obtained for 50 hybrid plants and ranged from 0 to 333 seeds per plant, with an average of 56 seeds per plant (Fig. 2). Nearly half (40%) of plants did not set seeds when bagged. No significant difference was observed between the two hybrid combinations J1C1 and J1C2 for number of self-pollinated seeds (ANOVA, $p < 0.05$).

The fertility of interspecific F_1 hybrid types AABC, BBAC, and CCAB was compared for bagged seed set and pollen viability. BBAC hybrids produced the highest number of seeds per plant, followed by AABC hybrids and CCAB hybrids. Interestingly, similar proportions of AABC and BBAC F_1 hybrids failed to produce any bagged seed, but fertile (at least one seed produced) BBAC F_1 hybrids produced factorially more seeds per plant on average than fertile AABC F_1 hybrids. CCAB F_1 hybrids were more likely to be pollen-sterile, set no bagged seed, and to produce fewer seeds when fertile when compared to AABC and BBAC F_1 hybrid types. Hybrid type was significantly associated with seed set and pollen viability (ANOVA, $p = 8.67 \times 10^{-13}$, $p = 6.23 \times 10^{-15}$, respectively, Tukey's HSD $p < 0.05$). BBAC F_1 hybrids were significantly different in the number of seed set compared to AABC and CCAB (Tukey's HSD $p < 0.05$). BBAC hybrids were also significantly different in percentage pollen viability compared to CCAB but not AABC hybrids (Tukey's HSD $p < 0.05$).

Fertility of AABC, CCAB, and BBAC interspecific S_1 hybrids

AABC, CCAB, and BBAC F_1 plants were bagged to encourage self-pollination to produce S_1 plants. The fertility of true AABC, CCAB, and BBAC S_1 hybrid

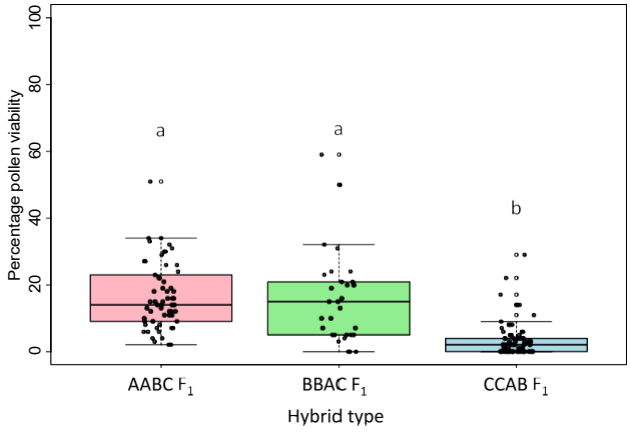

Fig. 1 Pollen viability estimates in first generation (F_1) interspecific *Brassica* hybrids with genome complements $2n$ = AABC, $2n$ = BBAC, and $2n$ = CCAB, derived from crosses between different genotypes of *B. napus*, *B. juncea*, and *B. carinata*. Different letters indicate significant differences between hybrid types (one-way ANOVA, $p < 0.0001$, followed by Tukey's Honest Significant Differences, $p < 0.0001$)

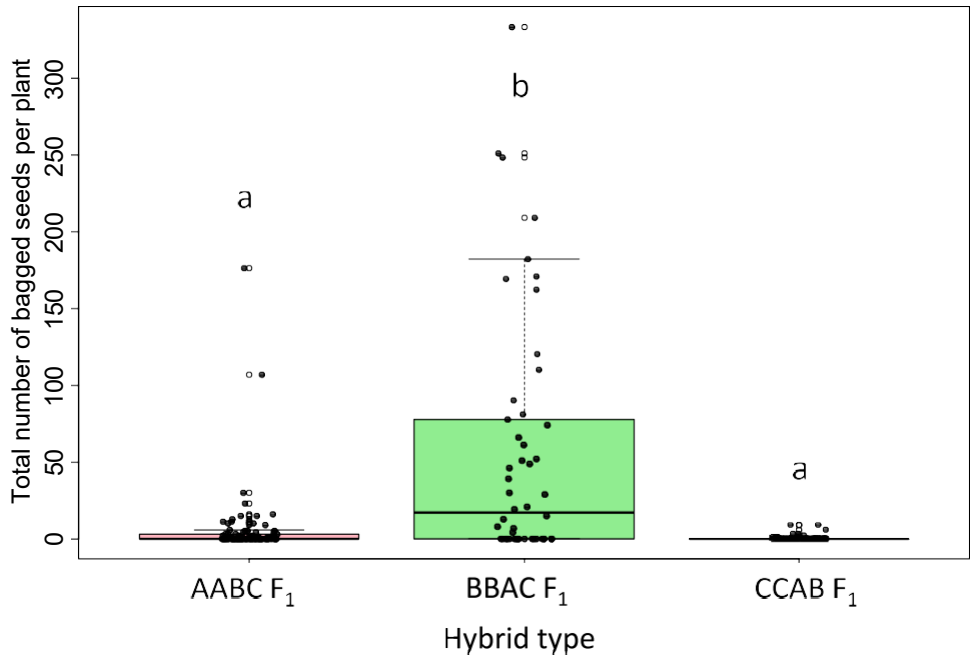

Fig. 2 Bagged seed set per plant produced in first generation (F_1) interspecific *Brassica* hybrids with genome complements $2n$ = AABC, $2n$ = BBAC, and 2n = CCAB, derived from crosses between different genotypes of *B. napus*, *B. juncea*, and *B. carinata*. Different letters indicate significant differences between hybrid types (one-way ANOVA, $p < 0.0001$, followed by Tukey's Honest Significant Differences, $p < 0.0001$)

plants was assessed by pollen viability and bagged seed production (Supplementary File S1e-g). For the 10 true AABC S_1 hybrids (four genotype combinations, resulting from four different *B. napus* parent genotypes crossed with one *B. juncea* parent genotype), pollen viability ranged from 0 to 93% with an average of 47% (Fig. 3). Bagged seed set data was collected for 9/10 true AABC S_1 hybrids and ranged from 0 to 182 seeds per plant, averaging 23 (Fig. 4).

Only five true CCAB S_1 hybrid plants were obtained from self-pollination of F_1 plants, all from different F_1 hybrid plants resulting from a single-genotype combination "N1C2." Pollen viability in the five plants ranged from 20 to 64%, with an average of 39% (Fig. 3).

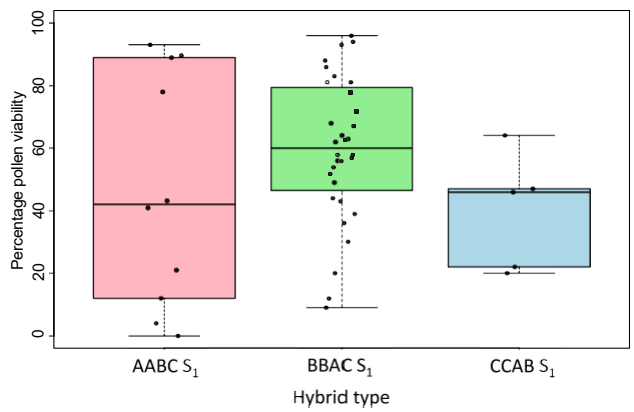

Fig. 3 Pollen viability estimates in second generation (S_1) interspecific *Brassica* hybrids derived from self-pollination of F_1 hybrids with genome complements $2n$ = AABC, $2n$ = BBAC, and $2n$ = CCAB, derived from crosses between different genotypes of *B. napus*, *B. juncea*, and *B. carinata*. No significant differences were observed between hybrid types (one-way ANOVA, $p > 0.05$)

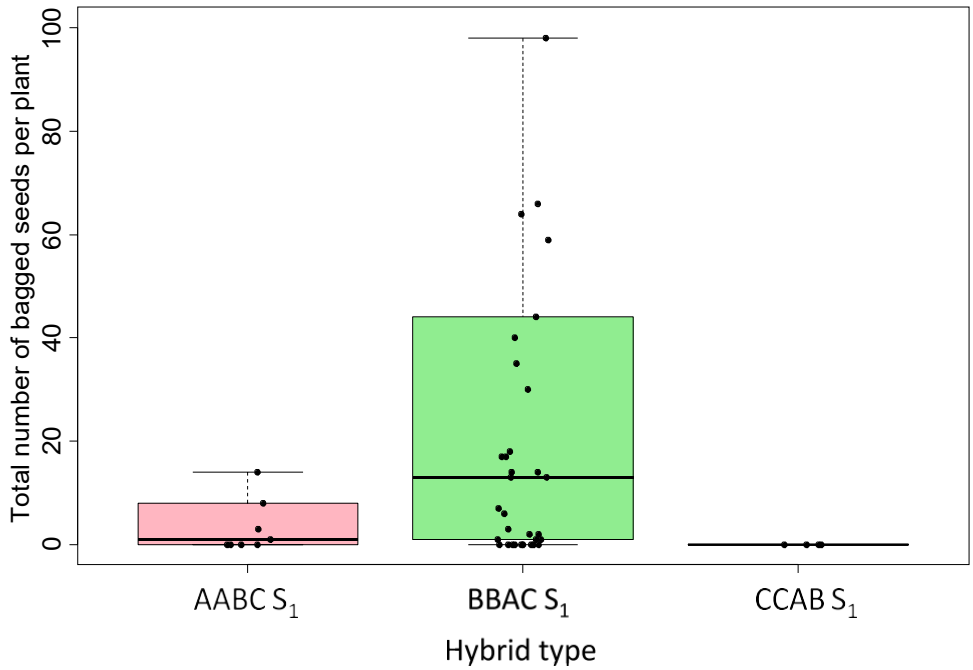

Fig 4 Bagged seed set per plant produced by second generation (S_1) interspecific *Brassica* hybrids derived from self-pollination of F_1 hybrids with genome complements $2n$ = AABC, $2n$ = BBAC, and $2n$ = CCAB, derived from crosses between different genotypes of *B. napus*, *B. juncea*, and *B. carinata*. No significant differences were observed between hybrid types (one-way ANOVA, $p > 0.05$)

However, none of the CCAB S_1 hybrids (only four were assessed) produced bagged seeds, indicating that these hybrids were completely sterile (Fig. 4).

A total of 38 true BBAC S_1 plants were assessed for fertility. Pollen viability was assessed in 32 out of 38 true hybrids, and ranged from 9 to 96%, with an average of 59% (Fig. 3). Bagged seed set was assessed in 37 out of 38 true BBAC S_1 hybrids and ranged from 0 to 403 per plant, with an average of 44 seeds/plant (Fig. 4). We observed significant differences in the number of bagged seeds produced per plant between the two different genotype cross combinations "J1C1" and "J1C2" (ANOVA, $p = 0.05$). A positive correlation was also observed between bagged seeds produced and pollen viability ($r = +0.48$).

The fertility of the three different S_1 hybrid types was assessed using self-pollinated seed set and pollen viability in order to determine whether fertility is affected by AABC, CCAB, and BBAC S_1 hybrid combinations or genotypes. Bagged seed set and pollen viability between AABC, BBAC, and CCAB S_1 hybrids were not significantly dif- ferent (ANOVA $p > 0.05$) (Fig. 3, Fig. 4). There was also no significant association between pollen viability or bagged seed set and genotype across all three hybrid types (ANOVA $p > 0.05$).

Chromosome counts in the AABC, BBAC, and CCAB S_1 hybrids

The chromosome numbers of 29 hybrid plants were counted in the S_1 generation (Supplementary File S1f-g). For the CCAB S_1 hybrids, 3/5 of the hybrid plants were analyzed, and had chromosome counts of 42, 43, and 44 chromosomes (Fig. 5). For BBAC S_1 hybrids, chromosome counts were done for 24 plants. The chromosome number ranged from 29 to 36 with a mean of 33 and a mode of 35 (8 individuals, the same as the BBAC F_1 parent). No chromosome information was obtained for true AABC S_1 hybrids.

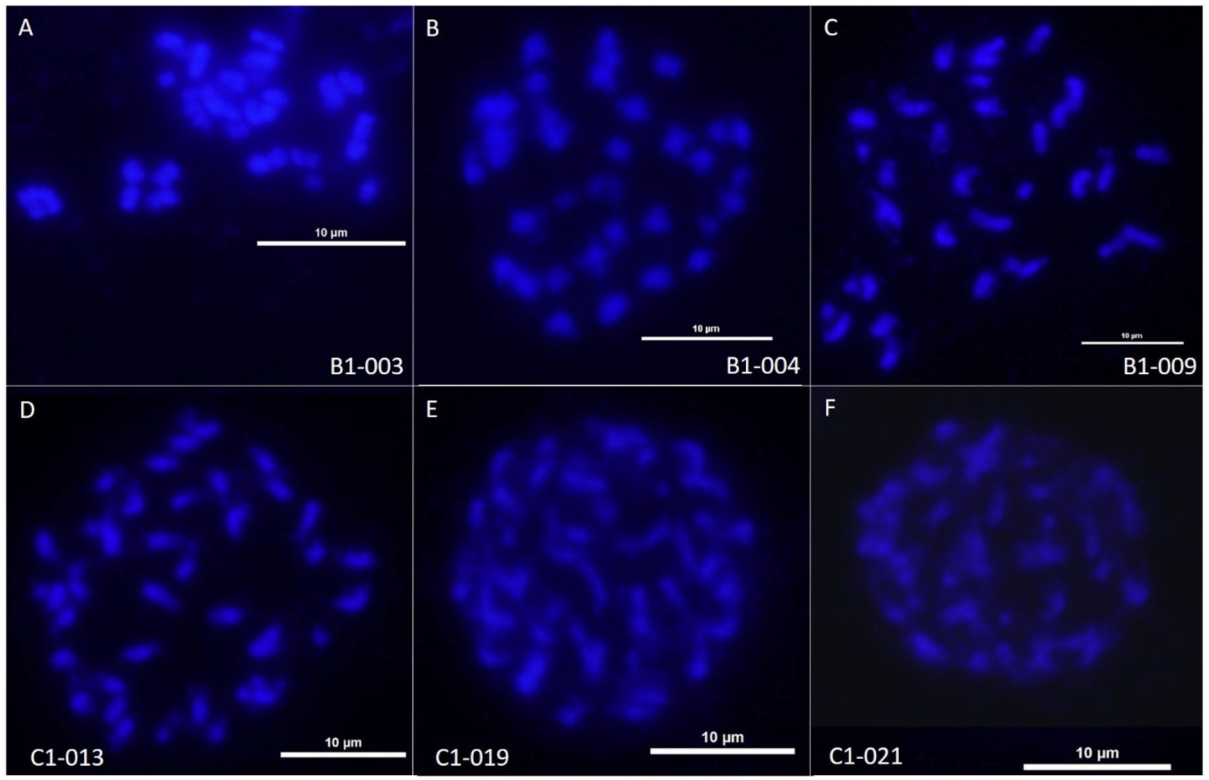

Fig. 5 Mitotic chromosome spreads of self-pollinated progeny resulting from first generation hybrids between *Brassica juncea* and *B. carinata* (BBAC S_1; **A**: B1-003, 2*n*~33 chromosomes; **B**: B1-004, 2*n*~36 chromosomes; and **C**: B1-009, 2n~34 chromosomes) and between *B. napus* and *B. carinata* (CCAB S_1; **D**: C1-013, 2*n*~43 chromosomes; **E**: C1-019, 2*n*~44 chromosomes; and **F**: C1-021, 2*n*~42 chromosomes)

Chromosome inheritance in AABC S_1 hybrids

A total of 10 true AABC S_1 hybrid plants had SNP genotyping information: five plants had A, B, and C genome data, and five plants had A and C genome data. For inheritance of the haploid B and C genome chromosomes across all ten individuals, we expected to see a 1:2:1 segregation ratio of 0 copies:1 copy:2 copies of each B- or C-genome chromosome (as a result of self-pollination of a parent AABC F_1 hybrid with one copy of each B- and C-genome chromosome). However, 0 C-genome chromosome copies were observed only 13% of the time, with statistically significant bias towards retention of C-genome chromosomes relative to the expected distribution (Pearson's χ^2 test, $p = 0.011$, Fig. 6). Upon closer inspection, seven out of ten individuals had one or two missing chromosomes plus one or two missing chromosome fragments (the latter indicative of non-homologous recombination events), while three individuals (A-01, A-02, and A3-001) inherited at least one copy of all C-genome chromosomes. B-genome data was only available for five individuals: A-01 and A-02 were not missing any B-genome chromosomes, A-65 was missing a copy of B1, A-71 was missing a copy of B7, and A-69 was missing three chromosomes completely (B4, B6, and B7) and was also missing most of chromosome B8, including the centromere.

Of the ten AABC S_1 hybrids, seven showed no significant deviation from the expected 50% heterozygosity for loci segregating for parental *B. napus* and *B. juncea* alleles in the A genome (Pearson's χ^2 test, $p > 0.05$). Three individuals deviated significantly from expected chromosome segregation ratios (Pearson's χ^2 test, $p < 0.0001$), all showing higher heterozygosity than expected: 86%, 89%, and 87% heterozygosity in individuals A3-001, A-01, and

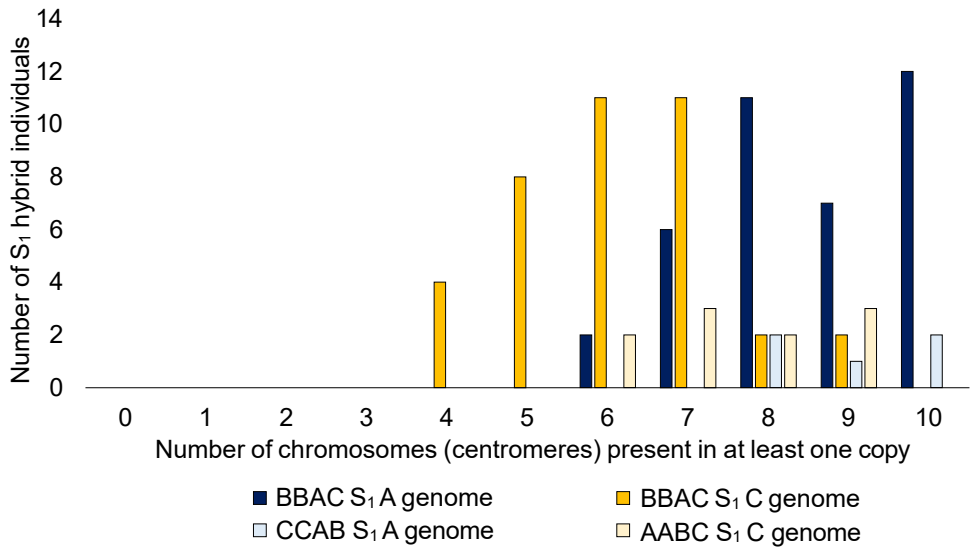

Fig. 6 Inheritance of chromosomes belonging to initially haploid A and C genomes in *Brassica* interspecific hybrids with genome complements AABC, BBAC, and CCAB in the first generation, followed by one generation of self-pollination to produce S_1 individuals. Dark blue and dark orange represent presence of A- and C-genome chromosomes in BBAC S_1 individuals, respectively, pale blue represents presence of A-genome chromosomes in CCAB S_1 hybrids and pale orange represents presence of C-genome chromosomes in AABC S_1 hybrids. Presence or absence of chromosomes was assessed by presence of the centromeric region based on SNP array genotyping

A-02. Surprisingly, three individuals from the combination J1N2 with expected 50% heterozygosity (Pearson's χ^2 test $p > 0.05$) also demonstrated some bias towards retention of N2 alleles over J1 alleles (30%, 37%, and 32% homozygous for N2 alleles, as compared to expected 25%), although this only reached significance ($p = 0.02$) for individual A-69.

Chromosome inheritance in BBAC S_1 hybrids

A total of 38 BBAC S_1 individuals were genotyped using the AC array: 20 from the genotype combination J1C1 and 18 from the genotype combination J1C2. Partial chromosome inheritance (a product of non-homologous recombination) was extremely common for the A and C genomes: on average, 40% of chromosomes showed this pattern (ranging from 5% of chromosomes for chromosome A08 to 68% of chromosomes for chromosome C9). Presence of a whole A- or C-genome chromosome was observed 54% of the time on average (ranging from 29% for chromosome C9 to 92% for chromosome A08), while complete absence of an A- or C-genome chromosome was observed only 6% of the time on average (chromosomes A03, A05, and C06 were always at least partially present, and chromosome C07 was completely lost most often, in 21% of individuals).

Inheritance of centromeric regions was used to assess presence of A- and C-genome chromosomes independent of non-homologous recombination events (partial chromosome presence) (Fig. 6). On average, based on centromere inheritance, A-genome chromosomes were lost 14% of the time and C-genome chromosomes were lost 32% of the time: both represent a significant deviation from the expected 25% retention of these chromosomes under a univalent segregation model (Pearson's χ^2 test, $p < 0.0001$ and $p = 0.003$, respectively). Based on an expected 75% chance of each chromosome being present (following self-pollination of a parent F_1 hybrid with one copy of each chromosome), chromosomes A04, A05, A06, and A08 were present more often than expected by chance (Pearson's χ^2 test, $p = 0.039$, $p = 0.0050$, $p = 0.039$, and $p = 0.0015$, respectively), and chromosomes C4 and C5 were lost more often than expected by chance (Pearson's χ^2 test, $p = 0.00037$ and $p < 0.0001$, respectively). Segregation of A- and C-chromosomes combined approximated the expected 25% inheritance of univalent chromosomes (Pearson's χ^2 test, $p > 0.05$).

Chromosome inheritance in CCAB S_1 hybrids

Only five true hybrid CCAB S_1 individuals were identified, and all were genotyped with the AC array. C1-019 showed no partial or complete loss of chromosomes, but at least one such event was observed for each other CCAB S_1 individual. Complete loss of chromosome A06 was observed in two individuals; no other chromosomes were lost completely although chromosomes A01 and A04 had undergone recombination events where only a small telomeric chromosome fraction was retained (in individuals C1-013 and C1-015, respectively). Large fractions of A-genome chromosomes were retained for events involving chromosomes A02 (in two individuals), A05, and A07.

Segregation of polymorphic loci in the C genome was surprisingly irregular: only one individual (C1-015) showed the expected 50% heterozygosity and approximately equal inheritance of alleles from the *B. napus* and *B. carinata* parent genotypes across all genomic loci (Pearson's χ^2 test, $p > 0.05$). One individual (C1-013) showed an excess of heterozygous loci (74%, Pearson's χ^2 test, $p < 0.0001$), while another individual (C1-019) showed an excess of homozygous loci (65%, Pearson's χ^2 test, $p = 0.002$). Individual C1-017 showed significant bias towards inheritance of *B. carinata* alleles over *B. napus* alleles (33% homozygous loci vs. 14%, Pearson's χ^2 test, $p = 0.005$), while three other individuals (C1-013, C1-019, and C1-021) showed the opposite trend, inheriting 18% vs. 8%, 40% vs. 25%, and 27% vs. 14% *B. napus* alleles relative to *B. carinata* alleles at homozygous loci (Pearson's χ^2 test, $p = 0.04$, $p = 0.06$, and $p = 0.04$, respectively).

Discussion

In this study, we analyzed fertility and chromosome inheritance in *B. juncea* × *B. napus* (AABC), *B. juncea* × *B. carinata* (BBAC), and *B. napus* × *B. carinata* (CCAB) F_1 interspecific hybrids and S_1 generation plants resulting from self-pollination of the F_1 hybrids. Although pollen viability in AABC and BBAC F_1 hybrids was similar and higher than that of CCAB F_1 hybrids (which were commonly male-sterile), only BBAC F_1 hybrids produced substantial numbers of true S_1 seeds. CCAB hybrids were all completely sterile by the S_1 generation, while AABC S_1hybrids were rescued from complete sterility by putative selection for unreduced gametes which transmitted complete sets of chromosomes from the F_1 parent: three such individuals were identified and all produced at least a few seeds. Chromosome inheritance in BBAC S_1 hybrids was biased towards retention of A-genome chromosomes and loss of C-genome chromosomes, and 40% of A- and C-genome chromosomes on average in these hybrids showed evidence of non-homologous recombination. Significant bias in allelic inheritance in the diploid A- and C-genomes of AABC S_1 and CCAB S_1 hybrids was also observed for several individuals, indicative of unreduced gamete involvement or other abnormal meiotic processes in both these hybrid types. As our experimental design mostly controlled for genotype-specific effects, our results suggest that fertility and viability of these hybrid lineages depend on genome structure, specifically on which genomes are present as haploid vs. diploid chromosome complements in the initial F_1 hybrids.

Our results suggest that the fertility and hence viability of interspecific hybrid lineages depend strongly on chromosome inheritance patterns in the first generations following the interspecific hybridization event, which are in turn dependent on initial genome structure in the hybrid. Interspecific hybridization is ubiquitous in plants, animals, and microorganisms and has the potential to generate a large amount of genetic diversity over a short period of time (Grant and Grant 1994; Mallet 2005; Arnold and Martin 2009; Stukenbrock 2016). However, hybrid lineages often suffer from poor fertility, as we observed for these *Brassica* hybrid types. The poor fertility of hybrids can serve as a barrier preventing their maintenance as independent populations, thus hindering their long term speciation potentials (Charron et al. 2019). Indeed, in our study a CCAB (*B. napus* by *B. carinata*) hybrid lineage completely failed to establish, with universally low fertility despite the generation of many F_1 hybrids from different genotype combinations. Different molecular mechanisms causing hybrid infertility exist, including genetic incompatibilities (nuclear and cytonuclear) and changes in genome architecture (ploidy number and chromosome rearrangements) (Rieseberg 2001; Maheshwari and Barbash 2011). Further physiological investigation of these hybrid types may be necessary to establish the exact mechanisms underlying the poor fertility observed.

We observed high levels of seed contamination resulting from inadvertent cross-pollination of the AABC and CCAB F_1 hybrids, with the majority of S_1 plants resulting from such inadvertent outcrossing, even under standard self-pollination conditions. Similar results were not observed for the more highly self-fertile BBAC S_1 hybrids, for which almost all S_1 progeny were pure. Contamination as a result of outcrossing to unknown parent may be a common fate of poorly self-fertile hybrids. In newly resynthesized *Brassica napus* hybrids, Katche et al. (2023) observed a 72% contamination rate using the SNP data analysis, which was attributed to possible outcrossing with established *B. napus* in the field where the hybrids were initially grown. Outcrossing of hybrids also often results in greater seed production than self-pollination (Schelfhout et al. 2008; Kumar et al. 2019). Possibly, inability to produce viable pollen, or failure of embryo development following self-pollination, results in strong selective pressure for otherwise rare cross-pollination events, resulting in high numbers of cross-pollinated progeny despite use of industry- and research-standard self-pollination methods in *Brassica*. Our results highlight the importance of using genome-wide markers to confirm parentage when working with highly infertile *Brassica* lines.

All our CCAB hybrids as well as most AABC hybrids were completely or nearly sterile in both the S_1 and F_1 generations, despite the presence of a complete diploid genome (CC or AA, respectively). Schelfhout et al. (2008) also observed substantially reduced F_1 sterility in *B. napus* × *B. juncea* interspecific hybrid crosses in both directions. The fertility of these hybrids did not increase following self-pollination, as only one plant was able to survive self-pollination from the F_3 to the F_4 generation. Choudhary and Joshi (2001) also found *B. juncea* × *B. napus* AABC F_1 hybrids to exhibit low fertility, which was attributed to chromosomal and genetic imbalance and/or cytoplasmic nuclear interactions. Similar results of low F_1 pollen and seed viability in hybrids produced between *B. juncea* and *B carinata* (BBAC) were reported by Kumar et al. (2019). Our *B. juncea* by *B. carinata* hybrids showed higher fertility, but this fertility was found to be dependent on the presence of least one copy of each homoeologous region from each of the A or C genomes in later generations (Katche et al. 2021). Inheritance of complete haploid genome complements in our AABC S_1 hybrids (as a probable result of unreduced gamete formation) also restored seed fertility. These results from our own and other studies suggest that a complete A or C genome resulting from the *Brassica* allotetraploid species *B. juncea*, *B. napus*, or *B. carinata* may no longer be sufficient for normal fertility in hybrids between these species. In support of this hypothesis, Pelé et al. (2017) also had trouble extracting the diploid A genome from *B. napus*, although a similar attempt to extract the A genome via intergeneric hybridization with *Isatis indigotica* followed by subgenome elimination was successful (Tu et al. 2010). Silencing and expression changes of subgenome-specific gene copies relative to their homoeologues have frequently been observed in *B. napus* (AACC) (Pan et al. 2019; Bird et al. 2021; Zhang et al. 2021; Wei et al. 2022); these changes may also negatively impact fertility after separation of the subgenomes in our interspecific hybrids.

In AABC, BBAC, and CCAB hybrids, frequent putative homoeologous exchanges involving the A and C genomes were detected. This high frequency of A/C pairing might have improved the chance of BBAC hybrids retaining at least one homoeologous gene copy from either of the A or C genomes, leading to improvement in seed fertility. In AABC, BBAC, and CCAB F_1 interspecific hybrids, Mason et al. (2010) found that B/C associations were detected in the lowest frequency followed by A/B associations, while A/C associations were most frequently formed in all hybrid types, with an average of 7 A-C pairs per cell in BBAC F_1 hybrids. In a report by Szadkowski et al. (2010), 30–47.5% A-C bivalents were observed in pollen mother cells of *B. napus* synthetics, with more than 10% of cells having more than three A-C bivalents. Similarly, high rates of recombination between the A and C genomes have been reported by other studies (Leflon et al. 2006; Szadkowski et al. 2010; Katche et al. 2021). These results can be explained by the genetic relationship between the A, B, and C genomes. Within the U's Triangle species, the A and C genomes have been shown to be more closely related to each other than to the B and will also pair more readily during meiosis (Nicolas et al. 2007, 2009; Attia and Röbbelen 1986; Udall et al. 2005, Udall et al. 2005; Gaeta et al. 2007; Mason et al. 2010).

Three AABC S_1 hybrids (A-01, A-02, and A3-001) and one CCAB S_1 hybrid (C1-013) in our study showed (1) inheritance of all or almost all

chromosomes from the haploid subgenome/s and (2) significantly elevated heterozygosity in the diploid (A or C) genome, consistent with involvement of unreduced gametes produced via a first division restitution-like mechanism. Unreduced gametes are known to be involved more frequently than expected by chance in crosses between species and to be produced at higher levels in interspecific hybrids (Ramsey and Schemske 1998; Mason and Pires 2015; Kreiner et al. 2017). In *Brassica*, unreduced gametes have been frequently observed in crosses between species and ploidy levels (Heyn 1977; Mason et al. 2011), as well as before in these specific hybrid types resulting from either cross-pollination events or microspore culture (Nelson et al. 2009; Mason et al. 2016). Unreduced gametes produced via a first division restitution-like mechanism (see (De Storme and Mason 2014) for review) inherit both homologous chromosomes from their parent, resulting in elevated heterozygosity, and inherit a copy of all univalent chromosomes. This appeared to confer a major advantage to seed fertility for these three unreduced gamete-derived AABC S_1 hybrids, which produced 3, 14, and 182 seeds, respectively, while only two other AABC S_1 hybrids produced seeds (1 and 8) and the other five AABC S_1 hybrids were sterile. The CCAB S_1 hybrid C1-013 did not produce any seed but also showed loss of most of chromosome A01, putatively due to a non-homologous recombination event. Another CCAB S_1 hybrid (C1-019) may have been the result of an unreduced gamete produced via second division restitution (whereby both sister chromatids are inherited) or some other abnormal meiotic process: this individual inherited a copy of each A-genome chromosome and showed significant bias towards loss of heterozygosity. However, C1-013 and C1-019 had an estimated 43–44 chromosomes, which are low relative to the expectation (CCAB = 37 chromosomes for a 2*n* gamete). This is difficult to explain via a self-pollination event, but may suggest that unreduced ovules developed directly into embryos, which has previously been documented in *Brassica* (Eenink 1974). It is possible that the individuals with high heterozygosity and retention of at least one copy of each haploid genome chromosome may have resulted from the combination of two reduced (normal) gametes; the observation of other bias towards inheritance of alleles from specific parents in other individuals supports this hypothesis (strong selection for rare viable gamete/embryo combinations). However, the division of the AABC S_1 hybrids into two groups, one group with retention of all haploid chromosomes and 86–89% heterozygosity in the diploid genome and one group with loss of chromosomes and/or chromosome fragments in the haploid genome and 41–54% heterozygosity, would seem to indicate two different meiotic processes were responsible for these two types of progeny. Further investigation is necessary to confirm this hypothesis, but our results suggest that involvement of unreduced gametes can boost success of interspecific hybridization events by increasing chromosome retention and hence fertility in otherwise infertile interspecific hybrids.

Significant bias towards presence of A-genome chromosomes and absence of C-genome chromosomes was observed in the BBAC S_1 hybrids. This bias was specifically associated with preferential (centromere) inheritance of chromosomes A04, A05, A06, and A08 in the A genome and preferential loss of chromosomes C4 and C5 in the C genome. These tend to be chromosomes which are less likely to be involved in A-C pairing, for which the most common pairs are A01-C1, A02-C2, A03-C3, and A07-C6 (Mason et al. 2014a). However, this trend did not include chromosome C7, which after A08 is the second most unlikely chromosome to be involved in A-C pairing (Mason et al. 2014b), also C7 was only 11% recombined in our study, relative to 5% in A08 and an average of 40% across all chromosomes. C4 and C5 also had high recombination rates in our study, at 50 and 60%, respectively. Together, these results suggest that there is a bias towards retention of A-genome chromosomes that is reduced or removed by non-homologous pairing with the C genome. However, there is no evidence for a similar bias towards loss of individual C-genome chromosomes (e.g. C7): the preferential loss of chromosomes C4 and C5 appears to be linked to their frequent non-homologous recombination with chromosomes A04, A05, and A06 (Mason et al. 2014a). Although similar biases towards inheritance of A-genome chromosomes or chromosome fragments over C-genome chromosomes or chromosomes fragments have been previously observed in *Brassica* allohexaploids (Gaebelein et al. 2019b) and in later-generation BBAC hybrids (Katche et al. 2021), the reason for this bias is still unknown. Gene-expression-based subgenome dominance is present in *Brassica napus* for up to a third of genes (Wei et al. 2022), and the *Brassica* A and C genomes show excellent dosage compensation (replacement of

function by the homoeologous gene copy; e.g., (Xiong et al. 2011; Samans et al. 2017; Gonzalo et al. 2019)), such that it seems unlikely that gene expression differences between subgenomes would have an immediate effect on gamete or embryo survival. However, in *Lolium* × *Festuca* interspecific hybrids, which show preferential loss of *Festuca* chromosomes, Majka et al. (2023) were able to attribute this preferential chromosome loss effect to subgenome-specific (*Lolium*-only) expression of kinetochore proteins controlling attachment of univalent chromosomes to microtubules, such that *Festuca* chromosomes were improperly attached and subsequently often lost as micronuclei following meiosis. Similar bias towards inheritance of *Allium roylei* over *A. cepa* chromosomes in *A. cepa* × *A. roylei* hybrids was attributed to female meiotic drive (Kopecký et al. 2022). Similar meiotic mechanisms may be operating in our BBAC S_1 hybrids, but further investigation would be required to confirm or refute this hypothesis.

Author contribution ASM, PVT, YTL, ZI, JZ, and DN performed experiments to produce F_1, S_1, and S_2 generation data. EK, EIK, and ASM performed data analyses, produced the figures, and drafted the paper. JB generated genotyping data, and EIK, PVT, JZ, and JB contributed to critical revisions of the manuscript. ASM conceptualized the project, supervised EK, PVT, EIK, YTL, ZI, and DN, and contributed to critical revisions of the manuscript. All authors approved the manuscript version for submission.

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4.0 Stable, fertile lines produced by hybridisation between allotetraploids *Brassica juncea* (AABB) and *B. carinata* (BBCC) have merged the A and C genomes.

4.1 Publication outline

The following publication describes our study on interspecific hybrids between *B. juncea* (2n = 4x= AABB) × *B. carinata* (2n = 4x = BBCC). F1 hybrids (2n = BBAC = 35) were self-pollinated for six generations while selecting for fertility. Meiotic pairing behavior improved from 68% bivalents in the F_1 to 98% in the S_5/S_6 generations, while initially low hybrid fertility also increased to parent species levels. The S_5/S_6 hybrids contained an intact B genome (16 chromosomes) plus a new, stable A/C genome (18-20 chromosomes) resulting from recombination and restructuring of A- and C-genome chromosomes. Our study presents the first experimental evidence that two allotetraploid species which share a common genome can come together in a hybridization to form a new, restructured genome.

4.2 Publication

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Research

Stable, fertile lines produced by hybridization between allotetraploids *Brassica juncea* (AABB) and *Brassica carinata* (BBCC) have merged the A and C genomes

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Summary

- Many flowering plant taxa contain allopolyploids that share one or more genomes in common. In the *Brassica* genus, crop species *Brassica juncea* and *Brassica carinata* share the B genome, with 2*n* = AABB and 2*n* = BBCC genome complements, respectively. Hybridization results in 2*n* = BBAC hybrids, but the fate of these hybrids over generations of self-pollination has never been reported.
- We produced and characterized *B. juncea* 9 *B. carinata* (2*n* = BBAC) interspecific hybrids over six generations of self-pollination under selection for high fertility using a combination of genotyping, fertility phenotyping, and cytogenetics techniques.
- Meiotic pairing behaviour improved from 68% bivalents in the F_1 to 98% in the S_5/S_6 generations, and initially low hybrid fertility also increased to parent species levels. The S_5/S_6 hybrids contained an intact B genome (16 chromosomes) plus a new, stable A/C genome (18–20 chromosomes) resulting from recombination and restructuring of A and C-genome chromosomes.
- Our results provide the first experimental evidence that two genomes can come together to form a new, restructured genome in hybridization events between two allotetraploid species that share a common genome. This mechanism should be considered in interpreting phylogenies in taxa with multiple allopolyploid species.

Introduction

Polyploidy is defined as the presence of more than two complete sets of chromosomes within an organism (Ramsey & Schemske, 1998; Soltis & Soltis, 1999). Polyploidy is reported to occur in many animals (e.g. fish, insects, and amphibians) and plants (e.g. fern and mosses), but with a higher frequency in flowering plants, and hence most major crops (Leitch & Leitch, 2008), where it represents a major mechanism of adaptation and speciation (Ramsey & Schemske, 1998). Reports indicate that 30–80% of all extant flowering plants are polyploids, with all angiosperms having experienced at least one round of whole genome duplication (Jiao *et al*., 2011). There are two major types of polyploids: autopolyploids, which arise within a population or species; and allopolyploids, which result from hybridization between two species.

In polyploid taxa, primary polyploids may also hybridize, leading to the formation of secondary polyploid hybrids (Rieseberg, 1997; Soltis & Soltis, 2009; Abbott *et al*., 2013). In the *Aegilops* genera, it was found that allotetraploids that share one common genome hybridize easily (Zohary & Feldman, 1962; Dubovets & Sycheva, 2017). In hybridization between allotetraploids that share a common genome, it has been suggested that this common genome serves as a buffer, providing the opportunity for recombination between the differential genomes and leading to the formation of many new variants of the recombinant genome (Zohary & Feldman, 1962; Kimber & Yen, 1988; Badaeva *et al*., 2002; Dubovets & Sycheva, 2017) (Fig. 1). Although never experimentally validated, polyploids with putatively recombinant genomes have been identified in the Triticeae tribe and in cereals (Wang *et al*., 2000; Badaeva *et al*., 2004; Moln´ar *et al*., 2013). The molecular analysis of genomic changes that accompany polyploidy has led to a significant breakthrough in understanding how primary polyploids form new, stable genomes. However, how secondary polyploids may form stable, recombinant genomes is unknown. Elucidating this process will deepen our understanding of micro-evolutionary differentiation within families and may assist in phylogenetic reconstruction.

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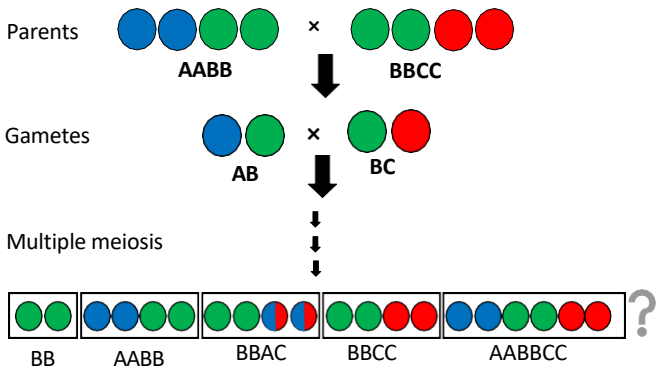

Fig. 1 Formation of a possible new, stable allopolyploid hybrid through hybridization between two allotetraploids that share one of two genomes in common. The different genome compositions BB, AABB, BBAC, BBCC, and AABBCC represent different possible karyotypes that could arise from this hybrid combination.

The *Brassica* genus is an important model for studying interspecific hybridization and polyploidy. It is a complex of related diploid and allopolyploid species containing the A, B, and C genomes, where the evolutionary relationship between six agriculturally important members of this genus was illustrated by U (1935). The 'triangle of U' consists of three diploid species (*Brassica rapa*, $2n$ = AA = 20; *Brassica nigra*, $2n$ = BB = 16; and *Brassica oleracea*, $2n$ = CC = 18) and three allotetraploids (*Brassica juncea*, $2n$ = AABB = 36, a product of hybridization between *B. rapa* and *B. nigra*; *Brassica carinata*, BBCC = 34, a hybrid between *B. nigra* and *B. oleracea*; and *Brassica napus*, $2n$ = AACC = 38, a hybrid between *B. rapa* and *B. oleracea*). The ancestral relationship that exists between the *Brassica* A, B, and C genomes has been well elucidated (Attia & R€obbelen, 1986; Lagercrantz & Lydiate, 1996; Mason *et al*., 2010; Chalhoub *et al*., 2014), with the A and C genomes shown to be more closely related to each other than to the B genome. Although the B genome species separated from the A/C lineage some 6 Ma, comparison of the palaeopolyploid genomes reveal extensive conservation of gene content and sequence identity (Navabi *et al*., 2013).

Brassica allotetraploids species can readily hybridize to produce trigenomic hybrids AABC, BBAC, and CCAB, with each hybrid combination having one of the subgenomes in a diploid state and the other two in a haploid state (Schelfhout *et al*., 2008; Nelson *et al*., 2009; Navabi *et al*., 2010; Mason *et al*., 2010). The chromosome pairing behaviour of trigenomic *Brassica* allotetraploid hybrids AABC, BBAC, and CCAB has previously been reported in the F_1 generation (Mason *et al*., 2010). Although all types of allosyndesis (A–B, B–C, and A–C) are observed in all hybrids at varying frequencies, AABC and CCAB hybrid types show little pairing between chromosomes belonging to the haploid genomes, whereas BBAC hybrids show high frequencies of A–C pairing (Mason *et al*., 2010). However, the fate of these hybrid lineages under self-pollination conditions in subsequent generations has never been reported. In this study, we aimed to determine the genome stability and fertility of BBAC hybrids across multiple generations, to see if stable, fertile hybrid offspring could be recovered in later generations, and if so by which mechanism(s).

Materials and Methods

Experimental plant material

Brassica trigenomic tetraploids with genome complement BBAC are the products of the cross between the two *Brassica* allotetraploid species *B. juncea* ($2n$ = AABB) and *B. carinata* ($2n$ = BBCC). The parental *B. juncea* genotype 'JN9-04', hereafter represented with the code J1, was crossed with two different *B. carinata* genotypes – '195923.3.2_01DH' and '94024.2_02DH', hereafter called C1 and C2, respectively – to generate two separate F_1 hybrid genotypes: J1C1 and J1C2 (Fig. 2; Supporting Information Dataset S1). In brief, self-pollination in each generation was encouraged by enclosing racemes in microperforated plastic bags, and the most fertile plants in each generation (two to five plants per genotype combination) were selected as parents for the next generation. The generations were labelled as 'F_1' for the initial BBAC hybrids, then S_1 to S_6 for the subsequent six self-pollination generations.

Molecular karyotyping using marker-based genotyping data

Leaf samples were collected in 2 ml microcentrifuge tubes and stored at —20°C until use. DNA was extracted for the S_3, S_5, and S_6 generation plants using the BioSprint 96 plant work station (Qiagen) according to the manufacturer's instructions (http://qiagen.com/), and for earlier generations (S_1 and S_2) using the Microprep method described in Fulton *et al*. (1995). Single-nucleotide polymorphism (SNP) genotyping was performed using the Illumina Infinium 90K *Brassica* SNP array (A, B and C

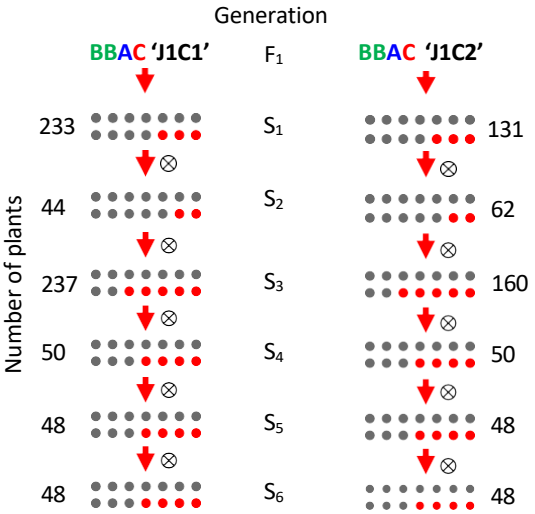

Fig. 2 Schema for generational selection of BBAC hybrid plants from the cross *Brassica juncea* 9 *Brassica carinata* based on highest numbers of self-pollinated seeds produced per plant. Red dots indicate the actual number of parent plants selected in each progeny generation.

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genomes) for the S_3, S_5, and S_6 generations, and using the Illumina Infinium 60K *Brassica* SNP array (A and C genomes) for S_1 and S_2 generations. Hybridization was performed according to the manufacturer's instructions for all samples, and the genotyped data were visualized and exported using GENOME STUDIO v.2.0.4 software (Illumina Inc., San Diego, CA, USA).

A total of 41 441 SNPs were exported for the A and C genomes after application of the recommended cluster file (Clarke *et al*., 2016) for S_5 and S_6 hybrids. A and C-genome SNPs were mapped to the Darmor-*bzh* v.8 reference sequence (Bayer *et al*., 2017) via BLAST of the SNP probe sequences (Karlin & Altschul, 1990). We filtered out SNPs that were mapped to the A genome but which amplified in *B. carinata* and SNPs that were mapped to the C genome but which amplified in *B. juncea*. As no allelic segregation is expected within the A and C genomes in these populations because they had no homologous pairing partners, SNPs that were heterozygous within the A and the C genomes were also filtered out. SNPs that had a 'no call' in > 10% of individuals within a haplotype block ($r^2 = 1$) of called SNPs or that had a 'call' in > 10% of individuals within a haplotype block ($r^2 = 1$) of 'no-call' SNPs were removed, in addition to SNPs showing patterns of segregation inconsistent with determined genomic locations. Genotype calls were finally converted to presence/absence calls (1 for presence and 0 for absence). After filtering, 26 484 SNPs were retained for the J1C1 genotype: 10 773 in the A genome and 15 711 in the C genome (Dataset S2). For the J1C2 genotype, 26 523 SNP markers were retained: 10 717 for the A genome and 15 806 for the C genome (Dataset S3).

The B genome SNP array data comprised 25 101 SNPs for which genomic positions were supplied with the public Illumina Infinium *Brassica* 90K array for an assembled *B. nigra* genome (available under MTA from Isobel Parkin, Agriculture and AgriFood Canada). The data were filtered to retain only SNPs that were polymorphic between the B genome of *B. juncea* and the B genome of *B. carinata* for each genotype combination (Datasets S2, S3). For early generations of BBAC S_1 and S_2 hybrids, SNP genotyping was performed using the Illumina Infinium 60K *Brassica* array and mapped to the Darmor-bzh v.8.1 reference sequence (Chalhoub *et al*., 2014). SNP filtering was performed as already reported herein (Datasets S4–S9). In summary, SNP genotyping and data analysis were performed for the S_1, S_2, S_3, S_5, and S_6 generations and for both the J1C1 and J1C2 lineages in each generation. The S_4 generation was not included because of a failure to collect leaf samples from S_4 hybrids.

The R package CHROMDRAW (Jane˘cka & Lysak, 2016) was used to produce the karyotypes of these hybrids. The centromere locations for the A and C genomes were assessed using the populations and methods reported in Mason *et al*. (2016) for *B. napus* Darmor-*bzh* v.1 (Chalhoub *et al*., 2014), remapped to the latest version of the *B. napus* cultivar Darmor-*bzh* v.8 reference genome sequence (Bayer *et al*., 2017) (Table S1).

Cytological analysis

Root tips and young flower buds were collected and prepared according to the procedure of Snowdon *et al*. (1997), and Leflon *et al*. (2010). Mitosis slides were observed after 4⁰,6-diamidino-2-phenylindole staining to visualize chromosomes under ultraviolet excitation using a Leica fluorescence microscope (Leica DMR; Leica Microsystems, Wetzlar, Germany) and meiosis slides were stained with 1% acetic acid carmine solution and observed using a Leica microscope with phase contrast. Mitotic chromosome analysis was done for 34 plants in the S_4 generation, 60 plants in the S_5 generation, and 82 plants in the S_6 generation. Two plants from each progeny set (eight plants per genotype) were selected to assess meiotic pairing behaviour at metaphase I of meiosis in the S_5 and S_6 generations. A minimum of 20 (mode 40) pollen mother cells from two different buds were assessed per plant for which data could be collected (Table S2).

Bacterial artificial chromosome–fluorescence *in situ* hybridization and genomic *in situ* hybridization

Slide preparation followed by hybridization using bacterial artificial chromosome (BAC)–fluorescence *in situ* hybridization (using BAC clone BoB014O06 containing C-genome-specific dispersed repeat sequence *Bot1* (Alix *et al*., 2008) labelled with Cy3) and genomic *in situ* hybridization (using DNA extracted from *B. nigra* labelled with fluorescein isothiocyanate) was carried out according to the procedures detailed in Leflon *et al*. (2006) and Mason *et al*. (2010). Images were captured using a Leica fluorescent microscope (Leica Microsystems).

Fertility data

Total seed set data was collected for all plants after encouraging self-pollination using micro perforated sleeves to enclose racemes. Newly opened flowers (at least two per plant) were collected when plants started flowering and pollen stained with 1% aceto-carmine solution before assessing viability using a Leica microscope. At least 300 pollen grains were counted per flower. Plants were then bagged to encourage self-fertilization, and total seed was counted after drying.

Results

Fertility in BBAC F_1 and S_1 hybrids

BBAC F_1 hybrids from two genotype combinations (produced between a homozygous inbred line of *B. juncea* with two doubled-haploid-derived lines of *B. carinata* – see (Mason *et al*. (2011b) for details) were grown under several different glasshouse and controlled-environment growth-room temperature conditions. The seed fertility under all conditions ranged from 0 to 333 seeds/plant with an average of 101 seeds/plant (Dataset S1; includes subset of plants from Mason *et al*., 2011b). Pollen viability was collected for a subset of individuals: F_1 hybrids showed moderate pollen production (average 15%, range 3–59%).

A total of 44 BBAC S_1 plants (20 J1C1 and 24 J1C2) resulting from seeds produced by F_1 hybrid parents were grown under

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glasshouse conditions, and a further 220 BBAC S_1 seeds (113 J1C1 and 107 J1C2) were grown in the field (200 direct sown, 20 germinated under glasshouse conditions and planted out at the four to six-leaf stage) (Table S3).

Forty of the 44 glasshouse-grown plants (four were not genotyped) were found to result from self-pollination of F_1 hybrids following genotyping with the Illumina Infinium *Brassica* 60K genotyping array, as expected. Fertility varied dramatically in the S_1 generation, with 9–96% pollen viability and 0–403 seeds per plant. Most field-grown BBAC S1 (J1C1 and J1C2 combined) plants (85%, 186/220) failed to produce any seeds, but most glasshouse-grown BBAC S_1 (73%, 29/40, as 4/44 plants were not SNP genotyped and were left out of the analysis) did produce seeds. Significant differences ($P < 0.05$, one-way ANOVA) were observed between the two genotypes of BBAC S_1 hybrid: only 6% of J1C1 plants (6/107 sown seeds) produced seeds under field conditions, as opposed to 25% of J1C2 plants (27/113). For glasshouse-grown plants, 10% of J1C1 (2/20) and 55% of J1C2 (11/20) with a combined average of 33% (13/40) failed to produce any seed.

Chromosome numbers, pairing behaviour, and genome constitutions in BBAC S_1 hybrids

Chromosome count data were obtained for 30 BBAC S_1 plants. An average of 33 chromosomes with a mode of 35 and a range from 25 to 36 chromosomes was observed. There were no significant differences between the two genotypes in terms of chromosome numbers of BBAC S_1 progeny (ANOVA, $P > 0.05$).

A high number of chromosomal rearrangements was observed in the A and C genomes, as assessed by deletions and duplications of parts of chromosomes based on SNP genotyping of 40 BBAC S_1 hybrids (two plants were discarded from the analysis because they were contaminated; Fig. 3). In the J1C2 population (20 plants), 53% of all A and C-genome chromosomes showed evidence of homoeologous recombination based on either absence or duplication of parts of chromosomes (0.53 events per chromosome per plant; Fig. 3d). More than one-third of A-genome and C-genome chromosomes were partially lost: 0.36 and 0.37 events per chromosome per plant for the A and C genomes, respectively. Complete loss of A and C genome chromosomes was relatively

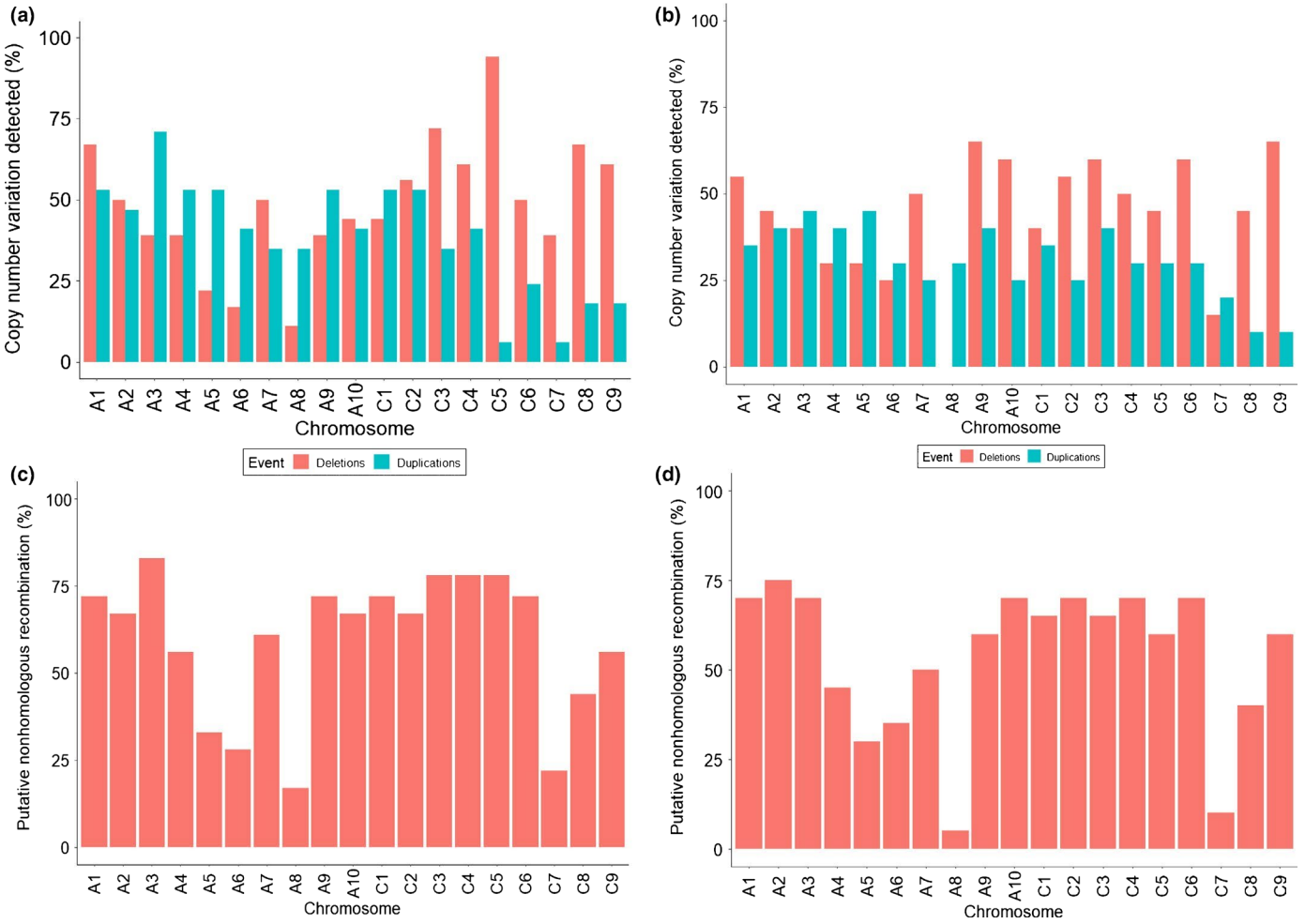

Fig. 3 Genetic changes in *Brassica juncea* 9 *Brassica carinata* self-pollinated S_1 interspecific hybrids as detected from Illumina Infinium *Brassica* 60K SNP array data. (a) Percentage of deletions/duplications (loss or gain of a whole or part of a chromosome) in the J1C1 population. (b) Percentage deletions/duplications in the J1C2 population for the different A and C chromosomes. (c, d) Percentage of individuals with recombinant chromosomes resulting from nonhomologous recombination events for each A and C-genome chromosome in the (c) J1C1 S_1 population and (d) J1C2 S_2 population.

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rare: seven losses of complete A-genome chromosomes and 12 losses of complete C-genome chromosomes were detected across the 20 plants (with 10 A chromosomes and nine C chromosomes per plant in the F_1), giving an average loss of 0.035 per chromosome per plant for the A genome and 0.066 for the C genome (Fig. 3b). Similar patterns were observed for the J1C1 lineage (18 plants, two discarded as a result of contamination), with 55% of A and C-genome chromosomes undergoing homoeologous exchanges (0.60 per chromosome per plant; Fig. 3c), and with few chromosomes completely lost (six events in the A-genome, 0.03 per chromosome per plant; and 17 in the C genome, 0.10 per chromosome per plant; Fig. 3a).

Selection and fertility in the BBAC S_2 generation

Self-pollinated seed set ranged from 0 to 745 seeds per plant in the BBAC S_2 J1C1 and J1C2 lineages. J1C1 plants set an average of 81 seeds, with 11% (5/44 plants) failing to produce any seeds. J1C2 had an average of 40 seeds/plant with 16% (10/62 plants) failing to produce any seeds. Only one plant in the J1C1 population failed to produce viable pollen, with an average of 71% pollen viability (range 0–97%) across the two populations. This result was obtained from 106 BBAC S_2 plants that were grown to maturity from selected S_1 parents and confirmed as true self-pollinated progeny.

Chromosome numbers and genomic constitutions in the BBAC S_2 hybrids

Chromosome counts were obtained for 22 BBAC S_2 individuals (11 J1C1 plants and 11 J1C2 plants) and showed a wide distribution (24–37 chromosomes, average 31). The chromosome distribution was the same in both lineages, with an average of 31 chromosomes. There was no observed correlation between chromosome number, pollen viability, and seed set (ANOVA, $P > 0.05$).

Nonhomologous recombination events were very frequent, and were differentially distributed across the different A and C-genome chromosomes (Fig. S1) in the J1C1 plants and J1C2 plants in the S_2 generation genotyped using the Illumina Infinium 60K *Brassica* array for the A and C genomes. Out of the 44 J1C1 plants that were SNP genotyped, five were unsuccessful; and for the J1C2 plants, 2/62 were unsuccessful. SNP analysis was therefore done for 39 J1C1 plants and 60 J1C2 plants. The average number of deletions per chromosome per plant was 0.6 in the J1C2 and 0.5 in the J1C1 progeny. The average number of duplications (gain of a partial or complete chromosome copy) per chromosome per plant was 0.36 in the J1C1 progeny and 0.38 in the J1C2 progeny. Chromosomes A8 and C7 consistently had the lowest number of deletion and duplication events in the J1C1 and J1C2 populations (Fig. S1a,b).

In the J1C1 population, chromosome C8 was most frequently lost, followed by A10, C7 and A4. In the J1C2 population, A8 was most frequently lost, followed by C7, A10, and C4. Chromosomes that showed partial deletions and/or duplication events were considered to have undergone a nonhomologous recombination event, as this is the primary mechanism by which deletions or duplications of only a partial instead of a whole chromosome can be observed (Mason *et al*., 2011b). Homoeology refers to chromosomes or chromosome segments that diverged from a common origin as a result of speciation and were brought back into the same genome by allopolyploidy, and which hence share sequence similarity. The sequence similarity between the A and C chromosomes, which is a measure of their homoeology, is reported in Chalhoub *et al*. (2014), Mason *et al*. (2014), and Lagercrantz & Lydiate (1996). The number of putative homoeologous exchanges in these S_2 hybrids were similar, especially for chromosomes with high homoeology between the subgenomes, and all chromosomes showed evidence of putative homoeologous exchange events. Chromosomes A6 and A8 had the lowest number of putative homoeologous exchanges in the J1C1 population, whereas A8 and C7 were lowest in the J1C2 population (Fig. S1c,d). Percentage genome presence was calculated using the number of present or missing SNPs for each chromosome in relation to the total number of SNPs for that chromosome.

Selection, fertility and genetic constitution of BBAC S_3-generation hybrids

Four BBAC S_2 plants (two from each of the J1C1 and J1C2 genotype combinations) were selected as parents of the BBAC S_3 generation. A total of 397 BBAC S_3 hybrid plants were grown and survived to maturity, with 100, 60, 93 and 144 plants from each BBAC S_2 parent. Of the total 397 plants, seed data were collected for 358 plants, with 39 being discarded due to heavy powdery mildew infestation. Seed production ranged from 0 to 1196 seeds/plant, with an average of 217 seeds/plant. Only 4% of plants (14/358) failed to produce self-pollinated seed (Dataset S1).

Of the total number of plants grown, 88 J1C1 plants and 92 J1C2 plants were SNP genotyped using the Illumina Infinium *Brassica* 90K SNP array (Table S3). Using the proportion of SNPs present and absent for each chromosome, we calculated the percentage of genome present for each chromosome. In comparison with the S_2 generation, the variation in the percentage of the genome present for the different chromosomes was reduced in the S_3 generation, as expected for increasing fixation of 'heterozygous' chromosome rearrangements. For example, chromosome A1 was present 30–100% of the time in the J1C1 S_2 generation and 60–75% of the time in the J1C1 S_3 generation, whereas chromosome A2 was present 0–100% of the time in the S_2 but from 0 to 25% of the time in the S_3. Although this type of variation was generally reduced in the S_3 generation compared with the S_2 generation, it was still high for some chromosomes, including A7, C3, C4, C6 and C7 of the J1C1 lineage. There was a significant difference in the percentage genome present between the chromosomes in both populations (ANOVA, $P < 0.05$; Fig. 4c,h).

There was no significant effect of any large-scale (> 0.5 Mbp) genomic rearrangements in the A and C genomes on fertility. However, stepwise regression analysis showed that the absence of

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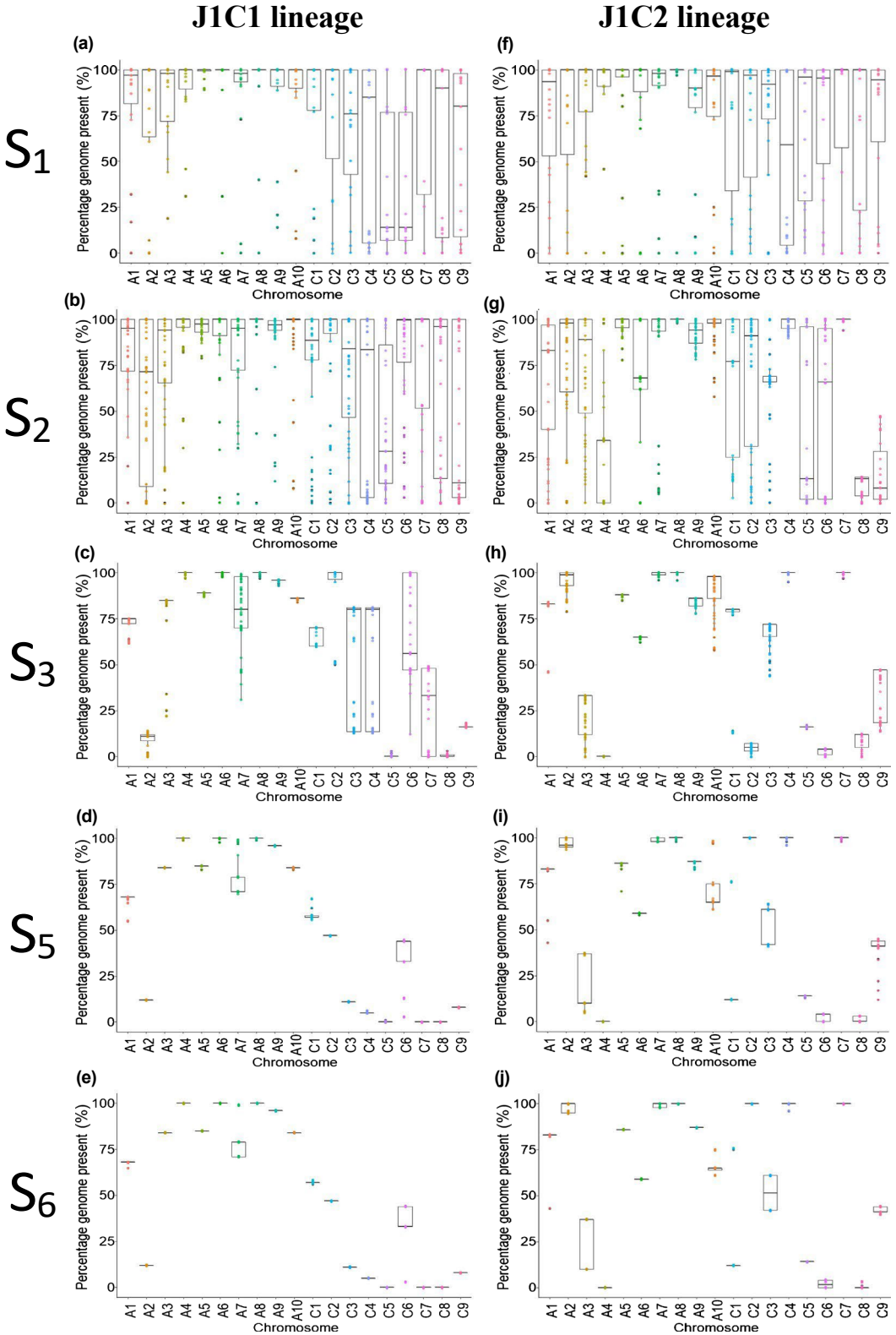

Fig. 4 Percentage of genome present per chromosome in *Brassica juncea* by *Brassica carinata* self-pollinated interspecific hybrids from the S_1 to S_6 generation (top to bottom) in (a–e) J1C1 hybrids and (f–j) J1C2 hybrids.

chromosome A3 in the J1C1 progeny and A1 in the J1C2 progeny reduced fertility ($P = 0.03$ for J1C1 and $P = 0.0122$ for J1C2). The B genome showed very limited genetic changes compared with the A and C genomes, indicating limited recombination between the B and A/C genomes. However, a 2.8 Mbp deletion at the top of chromosome B8 (loss of both copies),

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which was present in 19% of J1C2 hybrid plants, caused a reduction in seed fertility ($P = 0.00085$, stepwise regression analysis).

Fertility and chromosome numbers in the BBAC S_4 and S_5 hybrids

By the S_4 generation, there was an increase in the pollen and seed fertility of these hybrids. The average pollen viability of the J1C1 and J1C2 populations was 51% (range 17–89) and 91% (range 67–98), respectively, with a significant difference between these two genotypes (ANOVA, $P < 0.05$). The seed fertility ranged from 274 to 1551 for J1C1 and 442 to 1884 for J1C2, with an average seed fertility of 809 seeds/plant for the J1C1 and 1127 seeds/plant for the J1C2 populations, with a significant difference between the two populations (ANOVA, $P < 0.05$). This was based on analysis of 100 plants: 50 J1C1 and 50 J1C2. The average J1C2 S_4 seed fertility was higher than that of the *B. carinata* C2 parent, but the seed fertility of the *B. juncea* and *B. carinata* C1 parent was higher than the average of both lineages. The chromosome numbers of these plants ranged from 32 to 36 in both genotypes, with an average number of 34. Seed fertility in the S_3, S_4 and S_5 hybrid plants was higher on average in the J1C2 plants than in the J1C1 plants (ANOVA, $P = 0.01$; Fig. S2). The average fertility of the S_5 was also less than that of S_4. This is likely the result of severe disease pressure in the glasshouse, as the fertility of the parent genotypes was also seriously affected.

Fertility of BBAC hybrids increased with generational selection

Hybrids generally became more fertile across generations with selection (in each generation the most fertile individuals were selected as parents of the next generation) (Dataset S1). The general increase in seed production was, however, reversed in the S_5 generation due to severe disease pressure in the glasshouse (Fig. S3). By the sixth generation of self-pollination, some hybrid progeny sets had restored equivalent seed fertility to their parent species, with a combined average of 1072 seeds per plant (Fig. 5c; Dataset S1). In the J1C2 lineage, there was an increase in pollen viability from the F_1 generation to the S_4 generation, after which (S_4–S_6 generations) pollen viability levels were close to those of the parental controls. Variation between plants in pollen viability also decreased across the generations (Fig. 5b). In the J1C1 lineage, pollen fertility also increased from the F_1 to S_6 generation, although the increase was less consistent across the generations compared with the J1C2 lineage. The variation observed between plants was also higher in the J1C1 lineage than in that of the J1C2 (Fig. 5a).

Chromosome number in later generation hybrids was highly conserved and restored regular meiosis

Chromosome number per plant was counted in the S_1, S_2, S_4, S_5 and S_6 generations (Fig. 5d; S_3 data were not obtained). In the F_1 generation, the chromosome number was $2n$ = BBAC = 35, as expected from the union of haploid gametes from *B. juncea* (AB) and *B. carinata* (BC) (Mason *et al*., 2010). Variation in chromosome number was higher in the first four self-pollinated generations (S_1–S_4) than in the last two generations (Fig. 5d). In the S_5 generation, 64 plants were analysed: 47 (74%) had 36 chromosomes, 4 (6%) had 35 chromosomes, and 13 (20%) had a chromosome number of 34. Of the total 82 plants (41 J1C1 and 41 J1C2) analysed in the S_6 generation, 64 (78 %) had 36 chromosomes, 13 (16%) had 34 chromosomes, and 5 (6%) had 35 chromosomes. Fewer J1C1 plants, 28/41 (69%), showed $2n$ = 36 chromosomes compared with J1C2 plants 35/41 (86%).

Meiotic chromosome pairing behaviour was analysed in the F_1, S_4, S_5, and S_6 generations (Table S4), most comprehensively in the S_5 and S_6 (Table S2). The parent *B. juncea* and *B. carinata* genotypes showed 100% regular bivalent pairing (18 and 17 bivalents at metaphase I, respectively) with no univalent or multivalent chromosome pairing configurations observed (Table S2), as expected from established allopolyploid species. BBAC F_1 hybrids ($2n$ = 35) from the J1C2 genotype combination were previously reported to show only 68% of chromosomes involved in regular bivalent chromosome pairing per cell on average (3.1I + 11.7II + 2.3III + 0.2IV; Mason *et al*., 2010). In the S_4 generation (four plants assessed), 95% regular bivalent pairing was observed (Table S4). An average of 96% regular bivalent pairing was observed in the S_5 generation, and 99% regular bivalent pairing in the S_6 generation (Table S4). S_5-generation hybrids showed an average meiotic configuration of 0.34I + 17.16II + 0.14III averaged across progeny sets, with a maximum of four univalents and two multivalents per cell. S_6-generation hybrids showed a significant improvement over the S_5 generation in meiotic regularity as assessed by percentage bivalent formation across progeny sets ($P = 0.028$; Fig. 6b,d; Table S2). In the S_6 generation, hybrids showed average configurations of 0.2I + 17.8II + 0.03III averaged across progeny sets, with a maximum of two univalents and one trivalent observed per cell. Neither progeny set nor lineage in either generation significantly affected meiotic pairing configuration (average number of univalents, bivalents, and multivalents).

Highly rearranged karyotype structure in later generation *Brassica* BBAC S_5 and S_6 interspecific hybrids

High-quality SNPs from 96 S_5 and 96 S_6 plants were used to determine the karyotype structure of S_5/S_6 hybrids. From these marker data, karyotypes of the A, B and C genomes for the two lineages were produced (Figs 7, 8, S3, S4). Based on the SNP data, all 16 B-genome chromosomes were present and not recombined with any A or C genome chromosomes in the S_5 and S_6 generations (Fig. 7a,b). This result was also confirmed using genomic *in situ* hybridization on mitotic and meiotic chromosome preparations (Fig. 7c), where the expected eight bivalents resulting from the correct pairing of the 16 B chromosomes were always observed at metaphase I and these chromosomes segregated properly at anaphase I (with eight chromosomes on opposite poles) (Fig. 7d). The B genome was fixed for either *B juncea* or *B. carinata* alleles, with some regions of residual heterozygosity.

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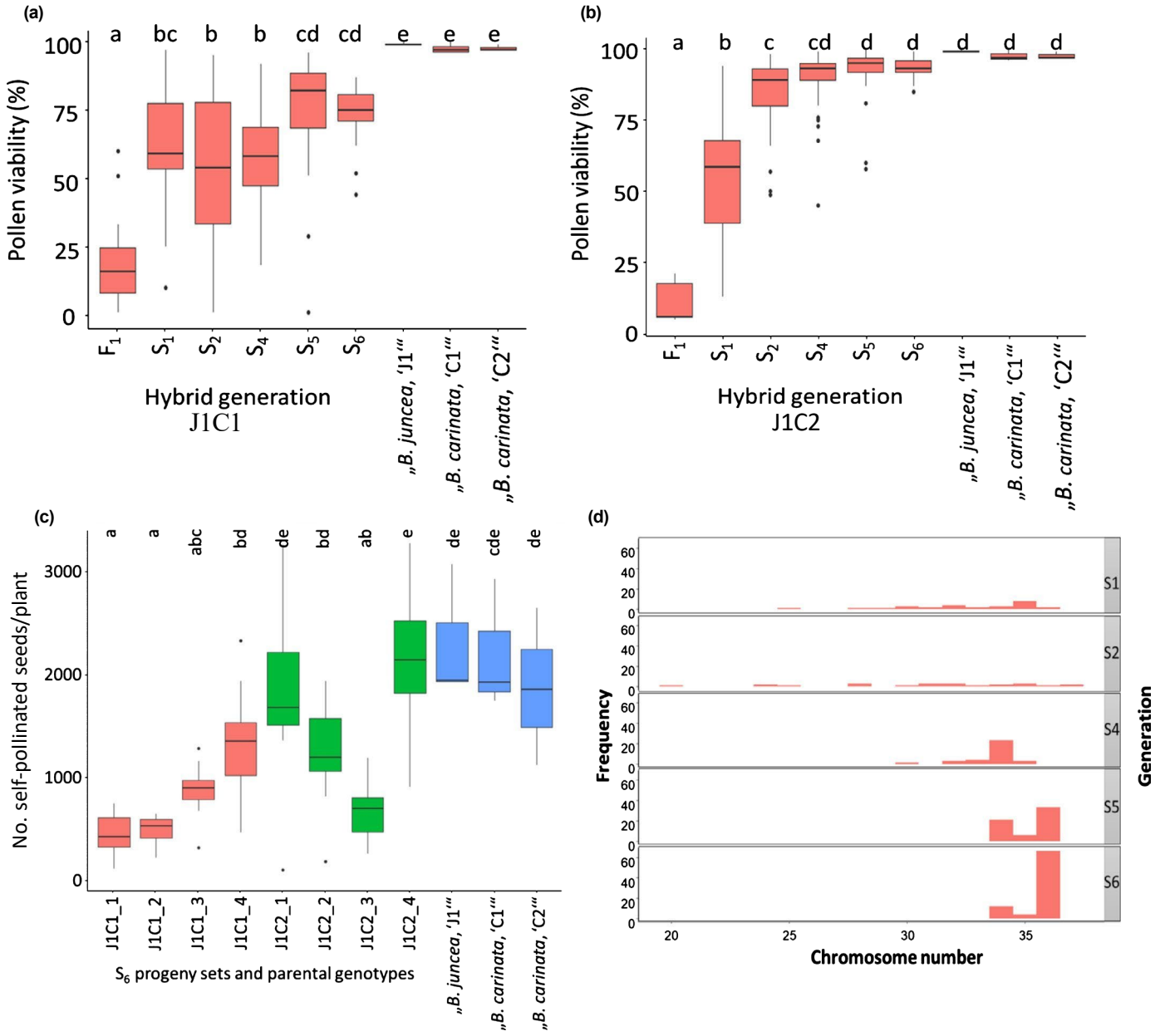

Fig. 5 Pollen, seed fertility, and chromosome number distribution of *Brassica juncea* 9 *Brassica carinata* interspecific hybrid progeny sets after six generations of self-pollination with selection for fertility relative to parental genotypes (*B. juncea* 'J1', *B. carinata* 'C1', and *B. carinata* 'C2'). (a, b) Pollen viability of (a) the J1C1 lineage and (b) the J1C2 lineage. Different letters indicate statistically significant differences. (c) Seed fertility of S_6 hybrids. Four progeny sets (12 plants per progeny set) are presented for each of two different genotypes J1C1 (green) and J1C2 (blue), along with the parental controls (red). (d) Distribution of chromosome number in self-pollinated progeny generations (S_1 to S_6) of *B. juncea* 9 *B. carinata* interspecific hybrids (F_1 = BBAC = 35 chromosomes) following selection for fertility in each generation.

By contrast, the A and C genomes were highly restructured, with chromosome losses and frequent translocations between the A and C genomes evident from the marker analyses. For the J1C1 lineage, 8/10 A-genome chromosomes showed genetic changes (deletions) based on SNP marker inheritance. whereas all C-genome chromosomes (9/9) showed genetic changes. Besides the loss of chromosome segments, there was no complete loss of any A-genome chromosome; for the C-genome, however, the three chromosomes C5, C7, and C8 were completely lost. These genetic changes were not different between the S_5 and S_6 generations. For the J1C2 lineage, 8/10 A-genome chromosomes were involved in rearrangements with a complete loss of chromosome A4 in the entire population, whereas 6/9 C-genome chromosomes were involved in rearrangements with a complete loss of chromosome C8. There was no clear selective pressure for particular chromosome segments or karyotype configuration: the genetic changes that occurred in the A and C genomes differed between the J1C1 and J1C2 lineages. For example, whereas in J1C1 all A-genome chromosomes were present, chromosome A4 was lost in J1C2.

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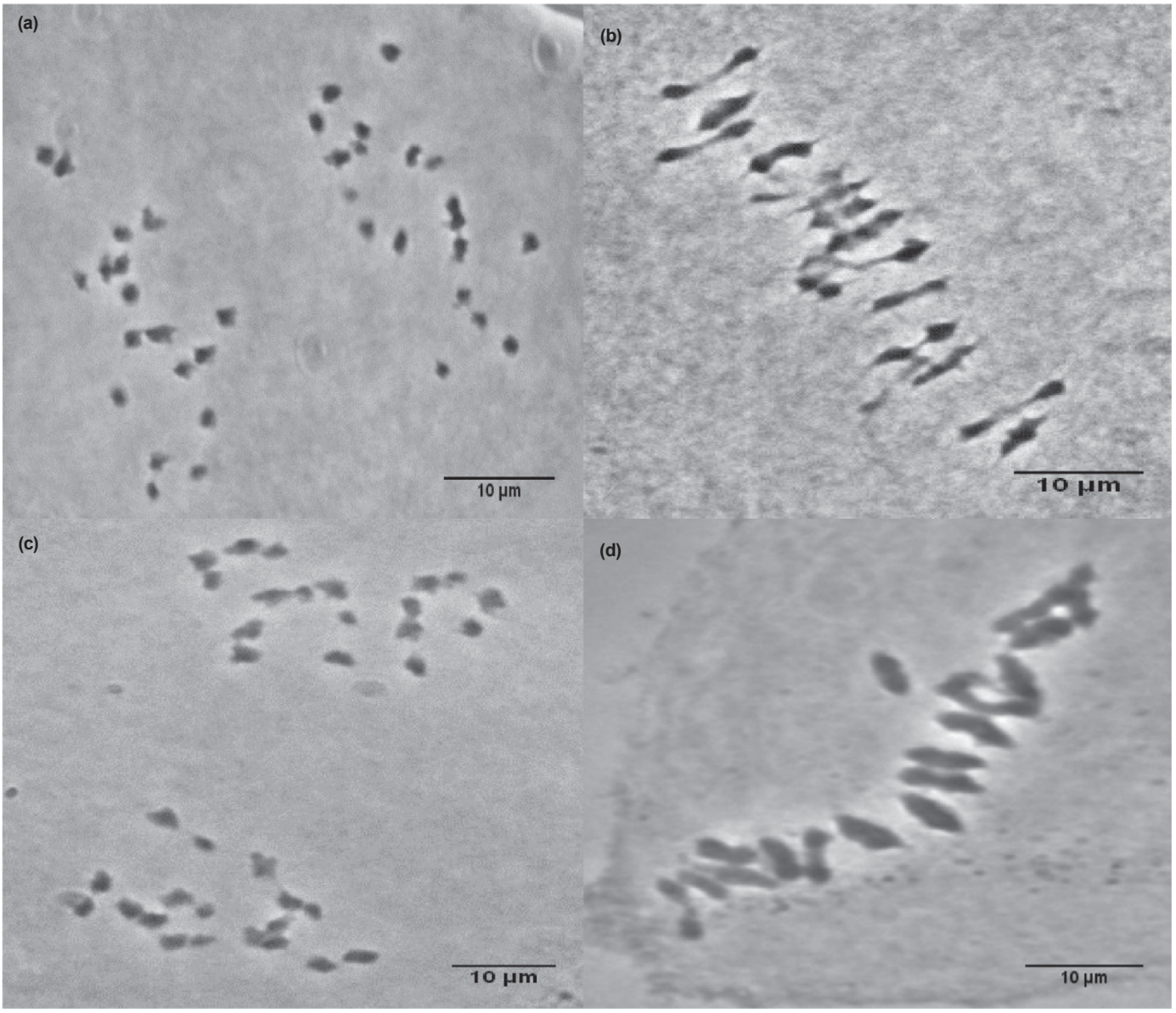

Fig. 6 Cytology of hybrids derived from the cross *Brassica juncea* 9 *Brassica carinata* followed by five or six generations of self-pollination with selection for high fertility. (a) Anaphase I in a BBAC S_5 hybrid; (b) metaphase I in a BBAC S_5 hybrid; (c) anaphase I in a BBAC S_6 hybrid; (d) metaphase I in a BBAC S_6 hybrid showing correct bivalent pairing and proper segregation.

Chromosome A5 recombined with chromosome C4 in J1C1, but with chromosome C5 in J1C2.

There was clear bias towards the retention of the A genome in both linages, with the C-genome homoeologues more frequently lost (Figs 4, 8). In the J1C1 population, 81–84% of the A-genome and 18–21% of the C genome was retained per progeny set by the S_6 generation, where all progeny sets had a common ancestor that retained 92% of the A genome and 58% of the C genome in the S_2 generation (Fig. S3). In the J1C2 population, 67–70% of the A genome and 42–51% of the C-genome was retained in the S_6 generation, where all progeny sets had an S_2-generation ancestor with 81% A genome and 75% C genome retention. Every homoeologous chromosome region was present in exactly one copy in the final modal A/C genome karyotypes in each lineage: either the A genome copy or the C genome copy of the homoeologous region was retained (Dataset S10). No homoeologous regions were observed in which both the A-genome and the C-genome copy were retained, or in which both the A-genome and the C-genome copy were lost.

Generational progression of chromosome changes

Most of the exchanges that took place between the A and C genomes occurred between the chromosomes with the highest degree of homoeology, such as A1/C1, A2/C2, A3/C3, A6/C7, A9/C9, and A10/C9 (Lagercrantz & Lydiate, 1996; Chalhoub *et al*., 2014; Mason *et al*., 2014). Most of the recombination events between the A and C genomes took place in the first hybrid

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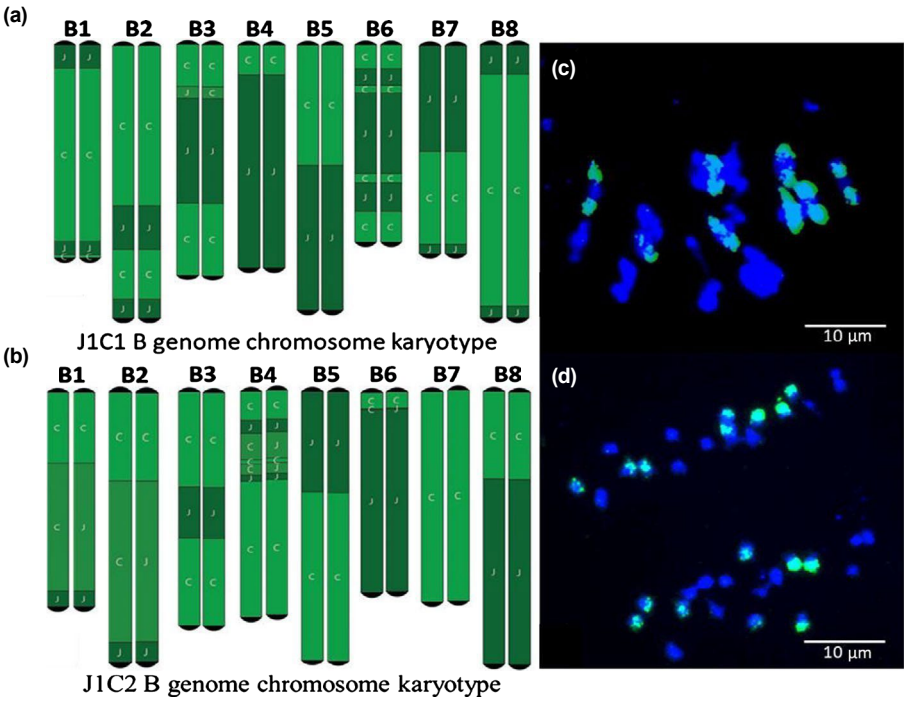

Fig. 7 Inheritance of B-genome alleles from *Brassica juncea* (J, dark green) and *Brassica carinata* (C, light green) based on single-nucleotide polymorphism marker genotyping in hybrids derived from the cross *B. juncea* 9 *B. carinata* following six generations of self-pollination (BBAC S_6) and selection for fertility: (a) J1C1 lineage and (b) J1C2 lineage. Forest green regions (CJ or JC) denote heterozygous regions with both *B. juncea* and *B. carinata* alleles. (c, d) Genomic *in situ* hybridization of (c) J1C1 chromosomes in metaphase I of meiosis with correct pairing of B genome (green) and (d) J1C1 chromosomes at anaphase I of meiosis showing proper segregation. Karyotypes of (a) and (b) produced using the R package CHROMDRAW.

meiosis (in F_1 plants), and hence were first observed in the S_1 generation in a heterozygous state (Figs S3, S4) before putatively being inherited and fixed in subsequent generations. Analysis of A-genome chromosome structure for J1C1 showed that genetic changes in chromosomes A1, A3, A5, A6, A7, and A9 remained the same between S_2 and S_6 generations. For the C genome, C4, C5, C6, and C7 did not undergo further changes between the S_2 and S_6 generations except for the loss of C8. Some of the karyotype rearrangements that were heterozygous in the J1C1 S_2 generation (e.g. involving A2, A3, and A7) appeared in the S_6 generation as homoeologous exchanges between A2/C2, A3/C3, and A7/C6 (Figs 8, S3, S4). Residual variation (presence of some individuals still segregating for chromosomal rearrangements or presence/absence) in karyotypes was observed in both lineages between progeny sets. In the J1C1 lineage, an A7/C6 translocation segregating in the S_5 generation was fixed in the S_6 with different variants between progeny sets, and a similar pattern was observed for an A2/C2 karyotype variant in the J1C2 lineage.

Discussion

In this study, we analysed the chromosome behaviour, stability, and fertility of *Brassica* trigenomic BBAC hybrids over six generations of self-pollination and selection for high fertility. Our results show that self-pollination and selection for fertility can lead to stable, fertile hybrids with novel karyotypes. Recombination and restructuring occurred between the A and C genomes in BBAC hybrids, whereas the B genome remained unchanged, and these A/C rearrangements appeared to be fixed by the $S_{5/6}$ generation, accompanied by a restoration of fertility and meiotic stability to produce 'true-breeding' progeny.

The ancestral relationship which exists between the *Brassica* A, B, and C genomes has been well-elucidated (Attia & Röbbelen, 1986; Lagercrantz & Lydiate, 1996; Ge & Li, 2007; Mason *et al*., 2010; Chalhoub *et al*., 2014), with the A and C genomes shown to be more closely related to each other than to the B genome. It has been predicted that the *B. nigra* (B) lineage diverged from the *B. rapa* and *B. oleracea* (A/C) lineage *c.* 7.9 million years ago (Mya) followed by the separation of the *B. rapa* (A) and *B. oleracea* (C) lineages *c.* 3.7 Ma (Inaba & Nishio, 2002; Panjabi *et al*., 2008). As a result of this close relationship, the A and C genomes pair readily with each other in haploids (Nicolas *et al*., 2009), AAC and CCA triploids (Leflon *et al*., 2006), synthetic allotetraploids (Xiong *et al*., 2011), and unbalanced AABC, BBAC, and CCAB tetraploid hybrids (Mason *et al*., 2010), whereas A−B and B−C homoeologous pairing is less frequently observed (Chen *et al*., 2005; Mason *et al*., 2011a; Navabi *et al*., 2010). We observed a complete lack of recombination between the B genome and the A genome and between the B genome and C genome in BBAC hybrids after six generations of self-pollination in our study. This observation is likely due to the fact that the B genome was present as homologous chromosome pairs, whereas the A and C genomes formed highly homoeologous pairing partners. Selection for fertility may also have selected against plants with homoeologous recombination events involving the B genome.

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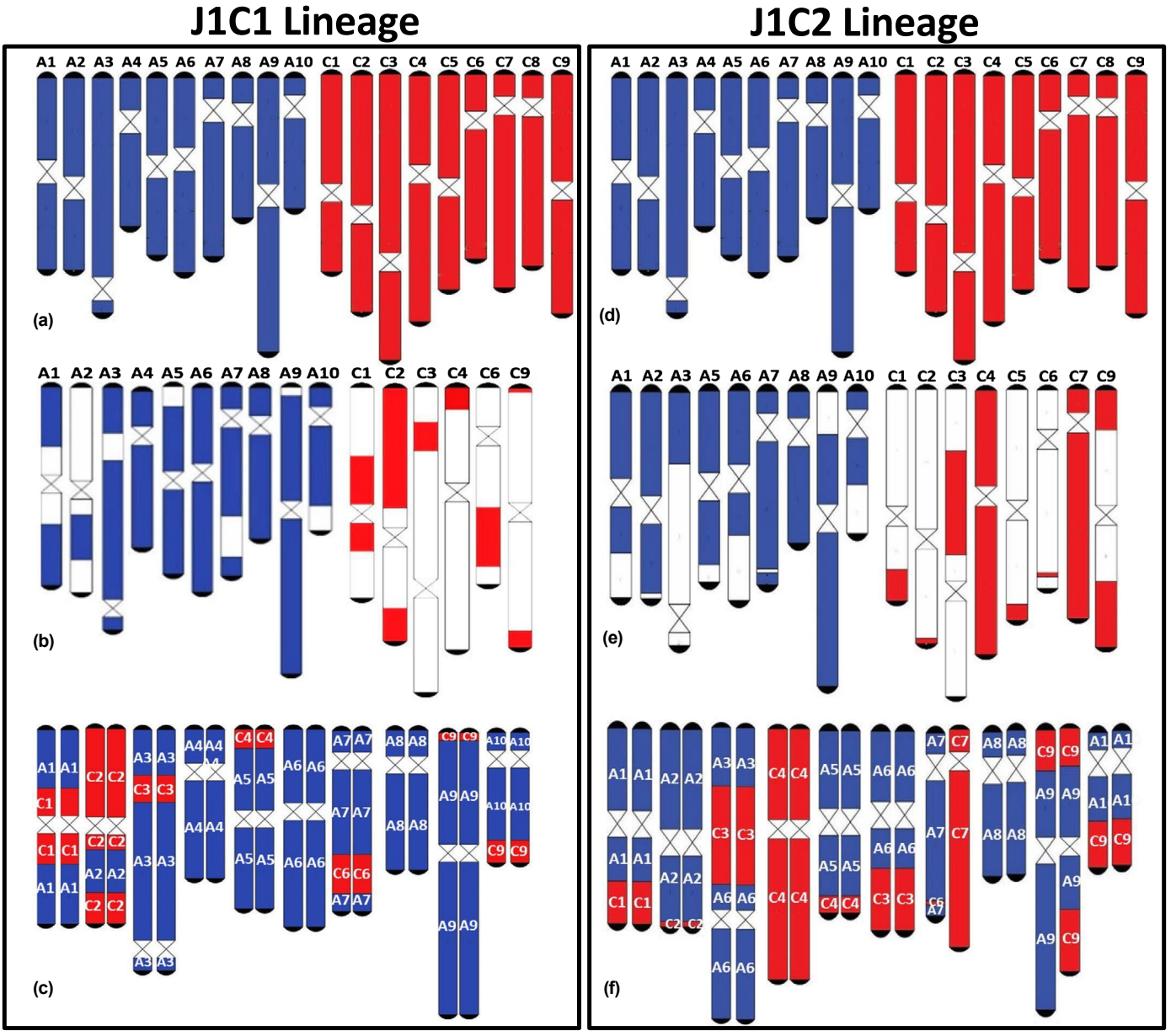

Fig. 8 Genetic changes and predicted recombined karyotypes in hybrids between *Brassica juncea* and *Brassica carinata* followed by six generations of self-pollination with selection for fertility (BBAC S_6) based on SNP marker genotyping: (a) A and C genome karyotype in the J1C1 BBAC F_1 hybrid with the expected 19 chromosomes given no recombination and segregation has occurred between the A and C chromosomes; (b) presence and absence of A and C genome chromosomes in the J1C1 lineage after six generations of self-pollination (S_6) based on SNP marker inheritance, where white represents absence of a chromosome segment: chromosomes C5, C7 and C8 are completely lost and therefore not represented; (c) the predicted modal recombined A–C chromosome karyotype for the J1C1 lineage after six generations of self-pollination (S_6) with a total of 20 chromosomes based on copy number analysis and chromosome counts; (d) A and C genome karyotype in the J1C2 BBAC F_1 hybrid with the expected 19 chromosomes because no recombination and segregation has occurred between the A and C chromosomes; (e) presence and absence of A- and C-genome chromosomes in the J1C2 lineage after six generations of self-pollination (S_6) based on SNP marker inheritance, where white represents absence of a chromosome segment: chromosomes A4 and C8 are completely lost and therefore not represented; and (f) the predicted modal recombined A–C chromosome karyotype for the J1C2 lineage after six generations of self-pollination (S_6) with a total of 20 chromosomes based on copy number analysis and chromosome counts.

Previously, self-pollination of a wheat (*Triticum aestivum*)−rye (*Secale cereale*) hybrid with $2n$ = RRAB up until the F_{17} generation revealed less frequent recombination between the A and B genomes and complete retention of the R genome in the early generations, but no restoration of genome stability (Dubovets & Sycheva, 2017). This is in contrast to our results of early fixation of karyotypes and restoration of genome stability by the $S_{5/6}$ generations. These contrasting results could be due to differences in the genetic control of meiosis between the wheat and *Brassica* genomes. The *Ph1* locus, located on chromosome 5B of bread wheat, is known to prevent homoeologous recombination between the wheat chromosomes almost entirely (Griffiths *et al*.,

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2006). Hence, the presence of the *Ph1* locus could have decreased the ability of the A and B genomes to recombine and stabilize in early generations. By contrast, no strong control preventing homoeologous recombination between the *Brassica* A and C genomes is expected.

Most genetic changes took place in the S_1/S_2 generation meiosis rather than in later generation meiosis in both lineages, as has previously been reported in other synthetic *Brassica* types (Prakash, 1999; Szadkowski *et al*., 2010). Interestingly, there was no clear selective pressure for particular chromosome segments or karyotype configurations between the two genotypes assessed. A similar lack of selection for particular chromosome segments has been observed in *Helianthus*, where the three homoploid hybrids *Helianthus anomalus*, *Helianthus deserticola*, and *Helianthus paradoxus* are all hybrids of two parent species *Helianthus petiolaris* and *Helianthus annuus*, but with different karyotypes in each hybrid species (Rieseberg, 2006).

The C genome was preferentially lost compared with the A genome in both BBAC lineages in our study. In allopolyploids, a phenomenon known as 'biased fractionation' is often observed over evolutionary time, whereby genes from one parental subgenome are preferentially lost (Bird *et al*., 2018; Emery *et al*., 2018). Biased fractionation has been reported in *Arabidopsis suecica* (Chang *et al*., 2010; Novikova *et al*., 2017), maize (*Zea mays*; Schnable *et al*., 2011), *Arabidopsis thaliana* (Thomas *et al*., 2006; Garsmeur *et al*., 2014), *B. rapa* (Wang *et al*., 2011), and cotton (Renny-Byfield *et al*., 2015). Differences in transposable element density and methylation and the possibility that certain phenotypic traits may largely be under the control of one subgenome could be responsible for biased fractionation, or for the preferential expression of genes from one subgenome (subgenome dominance) which may lead to biased fractionation (Cheng *et al*., 2016; Bird *et al*., 2018; Wendel *et al*., 2018). However, subgenome dominance does not seem to occur in all hybrid and polyploids; no subgenome dominance or evidence of biased fractionation has been observed in wheat, for example (Harper *et al*., 2016). In *B. napus*, Chalhoub *et al*. (2014) did not find any significant bias in gene expression towards the A or C subgenome, despite the fact that the C genome has a higher transposable element density and more methylation than the A genome, and hence would be predicted to be similarly expressed (Wendel *et al*., 2018). A more recent and comprehensive study by Wu *et al*. (2018) found a small but significant bias towards expression of genes from the A subgenome over the C subgenome (24% of gene pairs showed A > C compared with 15% showing C > A) in synthetic *B. napus*, but the generalizability of these results to natural *B. napus* is unknown. Interestingly, preferential loss of the C genome over the A genome has been observed frequently in different interspecific *Brassica* hybrid types, both for nonhomologous exchanges and whole chromosomes (Zhang *et al*., 2016; Samans *et al*., 2017), in line with our results. Possibly, the A genome contains more allelic variants responsible for improved fertility and viability than the C genome does, which would explain the retention of A-genome homeologues in our fertility-selected lines; more agriculturally significant quantitative trail loci also tend to be detected on the A genome relative to the C genome in natural *B. napus* (e.g. (Luo *et al*., 2017; Zou *et al*., 2018), supporting this interpretation.

Chromosome number was highly maintained within a narrow range from the BBAC F_1 hybrids ($2n$ = BBAC = 35) to the $S_{5/6}$ generation, by which generation almost all individuals showed between 34 and 36 chromosomes, accompanied by mostly regular meiosis. One important challenge that interspecific hybrids and neopolyploids encounter and must overcome to become established is the problem of incorrect meiotic pairing (Comai, 2005; Grusz *et al*., 2017; Pel´e *et al*., 2018), specifically between homoeologous chromosomes and chromosome segments belonging to different subgenomes. This problem has been shown to persist for several generations following allopolyploid formation in synthetic *B. napus* (Xiong *et al*., 2011), *Tragopogon* (Chester *et al*., 2012), and synthetic wheat (Zhang *et al*., 2013; Gou *et al*., 2018). Homoeologous chromosome pairing can result in loss of chromosomes and chromosome segments important for fertility and viability, accompanied by loss of the ability to produce 'true-breeding' offspring. It has been proposed that selection for increased fertility should stabilize the genome and reduce the frequency of aneuploid offspring (Tian *et al*., 2010). In initially unstable synthetic *Nicotiana* allotetraploid hybrids, the number of regular bivalents increased rapidly to > 99% after five generations of self-pollination (Ising, 1966). Similar observations have also been made in synthetic *Brassica* allotetraploids, albeit with genotype-specific variation (Song *et al*., 1995; Prakash *et al*., 2009). In our study, a combination of fertility-based selection and a high frequency of chromosome pairing between the homoeologous A and C-genome chromosomes may have interacted to retain viable chromosome complements.

A strong 'dosage compensation' effect was observed in the BBAC hybrids, as has previously been reported in *Brassica* (Xiong *et al*., 2011) and *Tragopogon* (Chester *et al*., 2012), where loss of A-genome chromosomes and homoeologous regions were compensated for by the retention of C-genome chromosomes and homoeologous regions and vice versa. Interestingly, we observed no instances in our S_6 generation hybrids where both the A and C genome homoeologue of a particular region were lost, or where both were retained. Only one copy (A or C) for each region of primary homoeology was detected for each of the modal karyotypes assessed in the J1C1 and J1C2 lines. This suggests that the negative effects of copy number variation (i.e. having an extra or missing copy of a homoeologous region relative to the normal dosage level of two copies (2A or 2C, as the third option for two copies of 1A + 1C is heterozygous/unstable and hence this was only observed in the early generations)) were extremely strong in these hybrids. As we applied very strong selection pressure for fertility in this project, we may have selected for lines with conserved dosages of A and C genomes. Aneuploidy can upset the expression levels of dosage-sensitive genes, resulting in lowered metabolic efficiency (Chester *et al*., 2012), and has also previously been linked directly to lowered fertility in *Brassica* allohexaploid hybrids (Gaebelein *et al*., 2019). Homoeologous chromosome copy numbers were also preferentially retained in self-pollinating lines of synthetic *B. napus*, suggesting that individuals with high deviation from chromosome balance had

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reduced fertility and were selected against during generational advancement (Xiong *et al*., 2011).

We conclude from this study that hybridization between *Brassica* allotetraploids sharing one of two genomes can lead to the formation of stable and fertile hybrids following self-pollination over a number of generations. Many authors have previously discussed the impact of homoploid and polyploid speciation and the various ecological, environmental, and genetic factors affecting their formation, maintenance, and diversification (Soltis & Soltis, 1999; Soltis *et al*., 2003; Mallet, 2005, 2007; Leitch & Leitch, 2008; Levin & Soltis, 2018). However, relatively few experimental studies have demonstrated pathways for homoploid and polyploid hybrid speciation. Using randomly amplified polymorphic DNA/intersimple sequence repeat markers, James & Abbott (2005) and Brennan *et al*. (2012) showed that *Senecio squalidus* is a homoploid hybrid formed by hybridization of *Senecio aethnensis* and *Senecio chrysanthemifolius*. Studies in wild sunflower suggest the homoploid hybrid *Helianthus anomalus* arose rapidly (within fewer than 60 generations) by hybridization between *Helianthus annuus* and *Helianthus petiolaris* (Ungerer *et al*., 1998). In allopolyploids, experimental studies have shown that the allotetraploids *Tragopogon mirus* and *Tragopogon miscellus* have formed repeatedly within the last 80 yr by hybridization of the three diploid species *Tragopogon dubius*, *Tragopogon pratensis* and *Tragopogon porrifolius* (Soltis *et al*., 2004; Chester *et al*., 2012; Lipman *et al*., 2013). In our study, experimental *Brassica* hybrids rapidly recovered correct chromosome pairing and maintained chromosome number, and some plants even produced more seeds than the parents. However, the genetic relationship between the genomes seems to be the main contributing factor leading to this result. The haploid genomes of these hybrids were highly restructured and behaved as homologous chromosomes with high levels of chromosome rearrangements. These hybrids could serve as a potentially important genetic resource that could be exploited for breeding purposes through transfer of A-genome introgressions via backcrossing into *B. carinata* or C-genome introgressions via backcrossing into *B. juncea*, and also support previously theoretical mechanisms of hybrid speciation (Mirzaghaderi & Mason, 2017; Levin & Soltis, 2018).

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Author contributions

EK performed all experiments to generate S_4–S_6 generation data, performed all data analyses, produced the figures, and drafted the paper. RG performed all experiments to generate S_3 generation data, and PV-T, Y-TL, ZI and DN performed experiments to produce S_1 and S_2generation data. JB generated genotyping data and contributed to critical revisions of the manuscript. PV-T also contributed to critical revisions of the manuscript. ASM conceptualized the project, supervised EK, PV-T, Y-TL, ZI and DN, and contributed to critical revisions of the manuscript. All authors approved the manuscript version for submission.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Fertility data collected for BBAC hybrids from the cross *Brassica juncea* 9 *B. carinata* (genotypes J1C1 and J1C2) and their parent controls over six generations of self-pollination.

Dataset S2 Illumina Infinium *Brassica* 90K SNP genotyping array data for the *Brassica* A- B- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' 9 *Brassica carinata* 'C1' hybrids in the fifth (BBAC S_5) and sixth (BBAC S_6) selfing generation.

Dataset S3 Illumina Infinium *Brassica* 90K SNP genotyping array data for the Brassica A- B- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' 9 *Brassica carinata* 'C2' hybrids in the fifth (BBAC S_5) and sixth (BBAC S_6) selfing generation.

Dataset S4 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' 9 *Brassica carinata* 'C1' hybrids in the first (BBAC S1) selfing generation.

Dataset S5 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' 9 *Brassica carinata* 'C2' hybrids in the first (BBAC S1) selfing generation.

Dataset S6 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' 9 *Brassica carinata* 'C1' hybrids in the first (BBAC S_2) selfing generation.

Dataset S7 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for

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Brassica juncea 'J1' 9 *Brassica carinata* 'C1' hybrids in the first (BBAC S_2) selfing generation.

Dataset S8 Illumina Infinium *Brassica* 90K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' 9 *Brassica carinata* 'C1' hybrids in the first (BBAC S_3) selfing generation.

Dataset S9 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' 9 *Brassica carinata* 'C1' hybrids in the first (BBAC S_3) selfing generation.

Dataset S10 Inferred primary homoeologous regions between the *Brassica* A and C genomes based on Illumina Infinium array single nucleotide polymorphism (SNP) genotyping.

Fig. S1 Genetic changes in *Brassica juncea* 9 *Brassica carinata* self-pollinated S_2 interspecific hybrids as detected from Illumina Infinium *Brassica* 60K SNP array data.

Fig. S2 Fertility of *Brassica juncea* 9 *B. carinata* interspecific hybrids in S_3, S_4 and S_5 generations of self-pollination with selection for fertility relative to their parent genotypes (*B. juncea* 'J1', *B. carinata* 'C1' and *B. carinata* 'C2').

Fig. S3 A- and C- genome presence and absence based on SNP marker genotyping in hybrids derived from the cross *B. juncea* 9 *B. carinata* (J1C1 genotype) following two generations of self-pollination (BBAC S_2) and selection for fertility.

Fig. S4 A- and C- genome presence and absence based on SNP marker genotyping in hybrids derived from the cross *B. juncea* 9 *B. carinata* (J1C2 genotype) following two generations of self-pollination (BBAC S_2) and selection for fertility.

Fig. S5 Number of genetic changes in different generations of *B. juncea* 9 *B. carinata* J1C1 and J1C2 interspecific hybrids relative to the F_1 generation.

Table S1 Putative centromere locations for the *Brassica* A- and C- genomes on the Darmor-*bzh* v8.1 reference genome estimated using half-tetrad analysis of mapping populations of *Brassica juncea* 9 *Brassica napus* (AABC) and *Brassica carinata* 9 *Brassica napus* (CCAB) hybrids.

Table S2 Meiotic chromosome pairing configurations of hybrids produced from the cross *Brassica juncea* 9 *Brassica carinata* followed by six generations of self-pollination (BBAC S_6) for two genotypes 'J1C1' and 'J1C2'.

Table S3 Seed information for self-pollinated *B. juncea* 9 *B. carinata* hybrids from S_1 – S_6 showing the number of plants which were grown in each generation and under which condition and the number SNP genotyped.

Table S4 Meiotic pairing of F_1, S_4, S_5 and S_6 generation hybrid plants.

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5.0 Discussion

5.1 Overview and scientific contribution of this thesis

In this thesis, I explored the meiotic chromosome behavior, inheritance and fertility of *Brassica* trigenomic interspecific hybrids with initial 2n = AABC, 2n = BBAC, and 2n = CCAB genome compositions formed by pairwise hybridization of *Brassica* allotetraploids *B. juncea* (2n = AABB), *B. carinata* (2n = BBCC) and *B. napus* (2n = AACC) in the F_1 and S_1 generations for all hybrid types, and for the BBAC hybrids, after self-pollination of these hybrids for six generations with selection for fertility. The main theme of this thesis was to establish if self-pollinating these unstable and unbalanced hybrids and selecting for fertility can lead to the recovery of stable fertile karyotpes in later generations. Genome stability, chromosome pairing, and fertility are vital aspect of newly formed hybrids and polyploids and therefore understanding the interplay between these and genetic factors responsible will further enlighten us about polyploidy and hybridization processes and how to harness these processes for agricultural improvement of crops. The results of this thesis show that selection for fertility can improve the meiotic stability and fertility of *Brassica* interspecific hybrids. However, the improvement in fertility and meiotic pairing behavior is related to the genome composition of the hybrid type and the genetic relationship of the genomes (Chapters 3 and 4). Interestingly, with the BBAC hybrids, we established that the A and C genome can recombine and behave like homologous pairing partners in the absence of actual homologous pairing partners. I show that there is extreme selective pressure for retention of at least one homoeologous region from each of the A and C genomes, altogether suggesting that the two subgenomes in the allopolyploids are quite interdependent on each other despite these being relatively recent allopolyploids (Chapter 4). CCAB hybrids however failed to establish and completely became infertile by the S_1 generation while AABC also being highly fertile were rescued from completely sterility by putative selection of unreduced gametes highlighting the importance of genome structure on fertility and viability of hybrid linages (chapter 3). The outcome of these studies may prove important when attempting to introgress useful traits from one *Brassica* species to another or creating new *Brassica* hybrid types. For recombination involving the A and C chromosomes, hybrids can be self- pollinated for several generations to increase the chances of recombination before backcrossing. For recombination involving the A/B and B/C early backcrossing will be important to avoid sterility upon self-pollination.

5.2 Meiotic control of chromosome pairing in interspecific hybrids.

Meiotic stabilization of newly created hybrids requires that homoeologous crossover formation is prevented while high levels of homologous crossovers are ensured (Nicolas *et al*., 2009; Gonzalo *et al.*, 2019). However, for stability to be achieved in these AABC, BBAC and CCAB trigenomic allotetraploid hybrids, homoeologous exchange between the haploid genomes will be necessary while the homologous chromosomes maintain pairing fidelity. Studies have shown that homoeologous pairing and meiotic instability is a common feature of newly created interspecific hybrids (Grandont *et al.*, 2014; Xiong *et al*., 2011; Mason *et al*., 2010; Cifuentes *et al*., 2010). How these initial unstable hybrids respond or change across generations: whether there is an improvement in meiotic stability has also been investigated. Studies carried out in different allopolyploid species have showed persistent karyotype instability over several generations. In wheat, persistent whole chromosome aneuploidy was reported to be associated with nascent allopolyploidy. Whole chromosome aneuploidy occurred ubiquitously in early generations (from self-generations S_1 - S_{20}) with variable frequencies (20 - 100%) (Zhang *et al*., 2013). Pedigree analysis showed no evidence of progressive karyotype stabilization even with multi-generation selection for euploidy (Zhang *et al*., 2013). Pollen viability was generally reduced by aneuploidy but the effect of aneuploidy was dependent on both the type of aneuploidy and the synthetic line (Zhang *et al.*, 2013). In synthetic *Brassica*, analysis of synthetic lines across 11 generations by Xiong *et al*., (2011) detected copy number changes in chromosomes in the S_0:S_1 generation which increased in subsequent generations. They observed reduced pollen viability and seed set with increasing number of chromosome dosage changes. Ninety-five percent of S_{10} : S_{11} resynthesized *B. napus* lines were aneuploid with a slight deviation of chromosome number from the expected 38. They concluded that selection against aberrant individuals with low fertility and those lacking control of homoeologous pairing may have been an important factor in the establishment of *B. napus* (Xiong *et al*., 2011). Even in natural populations *of Tragopogon*, Chester *et al*., (2012) uncovered massive and repeated number of chromosome variation with 76% of individual showing intergenomic translocations and 69% aneuploid for one or more chromosomes with no population fixed for a particular karyotype. However, 86% of plants showing aneuploidy still had expected chromosome number (Chester *et al*., 2012).

While the high number of homoeologous exchanges observed in the above studies parallels what is observed in this study and the complete sterility of AABC and CCAB hybrids, the early fixation of chromosome pairing and increasing seed and pollen viability observed in BBAC hybrids contrast what was obtained in the above studies even considering the fact that the initial F_1 hybrids were unbalanced. How can unbalanced hybrids lacking their homologous pairing partners stabilize within six generations accompanied by increasing fertility? For this to happen, the meiotic chromosome pairing must have been sorted out first.

How meiosis has adapted to cope with allopolyploids has only been deciphered in allopolyploid wheat and shown to be under the control of a single dominant locus, *Pairing homoeologous 1* (*Ph1*) located on chromosome 5B where a duplication of the *ZIP4* gene within the P*h1* locus prevents maturation of crossovers between non-homologous chromosomes (Martín *et al.,* 2014; Rey *et al.*, 2017). *ZIP4* is an essential factor for the main crossover pathway (called the class I or ZMM pathway) that also includes a set of critical proteins (e.g. MER2, MSH4, MSH5, SHOC1, HEI10 and PTD) in plants (Gonzalo *et al*., 2019). Wheat lacking *Ph1* accumulates extensive rearrangements and eventually become infertile (Sánchez- Morán *et al*., 2001; Greer *et al.*, 2012). Identifying genetic factors responsible for meiotic regulation in *Brassica* has also been a subject of research. One such locus is *PrBn* which was identified by (Jenczewski *et al*., 2003) and shown to affect pairing frequencies between the A and C genomes. Liu *et al*., (2006) mapped *PrBn* to Bna C09 and showed that *PrBn* displays variable expressivity. Three to six minor QTLs also have slight additive effects on the amount of pairing at metaphase I but do not interact with *PrBn* (Liu *et al*., 2006). However, both AACC parental lines had regular bivalent pairing, making the role of this locus in controlling homoeologous recombination unclear. By using both cytogenetic observations and high throughput genotyping to quantify the levels of homoeologous recombination in a segregating *B. napus* mapping population, Higgins *et al*., (2021) identified three QTL contributing to the to the control of homoeologous recombination in *B. napus.* One major QTL on BnaA9 contributed between 32 - 58% of the observed variation (Higgins *et al*., 2021). Five genes underlying BnaA9 were also identified with genes such as *RPA1C* (Replication protein A 1C) and *MUS81* (*MMS* and UV sensitive 81). While most studies have been carried out in euploid

Brassica plants, a detailed comparison of meiosis in near isogeneic allohaploid and euploid plants showed that the mechanism(s) promoting efficient chromosome sorting in euploids is adjusted to promote crossover formation between homoeologues in allohaploids. This suggests that in contrast to other polyploid species, chromosome sorting is context dependent in *B. napus* (Grandont *et al.*, 2014). This might help explain the pairing behavior of our hybrid where one of the genomes is present in a diploid number and the other two genomes in haploid copies. This helped encouraged chromosome pairing between the haploid genomes while the diploid chromosome chromosomes maintained pairing fidelity. In wheat and oat, the sorting of homoeologous chromosomes in euploids is paralleled by the almost complete suppression of CO formation between homoeologues in the corresponding allohaploids (Riley *and* Chapman. 1958; Gauthier & McGinnis, 2011). In these plants even if chromosomes have no choice but to recombine with their homoeologues, they are prevented from doing so by loci responsible for the cytological diploidization of euploid forms (Griffiths *et al*., 2006; Greer *et al*., 2012). Grandont *et al.*, (2014) showed that the situation is strikingly different in *Brassica napus.* Homoeologous synaptic and chiasmatic associations which are suppressed in euploids become dominant in allohaploids. This indicates that the mechanisms responsible for the early sorting homoeologous chromosomes in euploids *B. napus* does not suppress CO formation between homoeologous chromosomes in allohaploids (Grandont *et al.*, 2014). However, to what extent these allohaploids pair and recombine still depends on the genetic relationship of the allohaploids (Mason *et al.*, 2010). In BBAC hybrids, in the absence of homoeologous pairing partners, the A and C genomes pair with each other to form stable hybrids by the sixth generation. Why was the case different for the CCAB which became sterile by the S1 and AABC hybrids which almost became completely infertile? Besides the genome structure, genotype has been shown to affect the rate of allosyndensis in interspecific hybrids (Thomas & Al-Ansari, 1988; Jenczewski *et al.*, 2003; Mason *et al.*, 2010; Mwathi *et al.*, 2017). However, a sufficiently large number of genotypes were used in these hybrids. Therefore, genotype might not have played a significant effect on meiotic chromosome pairing compared to the genome structure and the genetic relationship of the haploid genomes. Within the U's Triangle species, the A and C genomes have been shown to be more closely related to each other than to the B and will also pair more readily during meiosis. B-A/C pairing are infrequent in all hybrid types relative to A-C (Nicolas *et al.* 2008; Attia and Röbbelen, 1986; Udall *et al*., 2005, Udall *et al.*, 2005; Gaeta *et al.*, 2007; Mason *et al*., 2010).

It is also possible that genetic factors in the B genome may be responsible. This will however need to be investigated.

Poor fertility is associated with loss of chromosomes in interspecific hybrids. Whole chromosome aneuploidy and structural alterations occur frequently in the new hybrids and allopolyploids while natural species maintain stable chromosome numbers (Zhao *et al*., 2021). The loss and gain of chromosomes has been shown to frequently involve homoeologous chromosome replacement and compensation, and chromosome numbers are frequently maintained at or near the euploid levels possibly due to dosage balance requirements (Xiong *et al.*, 2011; Zhang *et al.*, 2013). In resynthesized *Brassica* allohexaploids, Mwathi *et al*., (2017) observed low fertility which was associated with high levels of chromosome loss. In wheat, Zhang *et al.*, (2013) investigated trangenerational chromosome variation in synthetic allohexaploid wheat. They found that whole chromosome aneuploidy occurs ubiquitously in early generations from S_1 to > S_{20}) of wheat allohexaploids with a highly variable frequency (20% to 100%). Profiling of traits directly linked to reproductive fitness showed that pollen viability was greatly reduced by aneuploidy. The adverse effect of aneuploidy on seed set is dependent on both aneuploidy type and synthetic line. This persistent aneuploidy has also been reported by other studies in allotetraploid *Brassic*a (Xiong *et al*., 2011) and in *Tragopogon* (Chester *et al*., 2012). These studies contrast results from our present study where aneuploidy (loss and gain of chromosomes) was rare and chromosome number variation highly maintained. This was accompanied by increased bivalent pairing resulting in karyotpes rapidly achieving stabilization in BBAC lineages. One possible explanation for this is that the high rate of homoeologous pairing between the A and C genomes which caused them to behave as homoeologues could have prevented the loss or gain of chromosomes. Our results are however in line with studies reported by Tian *et al.*, (2010) where selection of hexaploid chromosome number across generations led to an increase in pollen viability of hexaploid *Brassica* hybrids. In the *Triticum-Aegilops* group Ozkan and Feldman, (2009) studied 18 newly synthesized allopolyploids in the S_1, S_2 and S_3 generations at different ploidy levels. They showed that bivalent pairing at first meiotic metaphase was enhanced and seed fertility was improved during each successive generation. A positive linear correlation was found between increased bivalent pairing, improved fertility and elimination of low copy no-coding DNA sequences. They concluded that rapid elimination of low copy

non-coding DNA sequences from one genome of a newly formed allopolyploid, and different sequences from different genomes is an efficient way to quickly augment the divergence between homoeologous chromosomes and thus bring about cytological diploidization (Ozkan and Feldman, 2009).

We observed that the C genome was more often lost compared to the A genome. Similar results have been obtained in studies involving resynthesized *Brassica* allotetraploids. (Rousseau-Gueutin *et al*., 2017) studied 33 resynthesized *B. napus* individuals from two open pollinated populations and found that meiosis was highly affected. Their genomes were deeply reshuffled after allopolyloidization with up to 8.5% of the C genome and 3.5% of the A genome deleted in only two generations. Similar results were reported by Gaebelein *et al*. (2019) in *Brassica* allohexaploids and Samans *et al*. (2017) in synthetic *B. napus*.

5.3 Limitations and unanswered questions

This study about meiotic stability and chromosome pairing behavior in *Brassica* trigenomic hybrids may be seen to have some potential limitations. The first potential limitation of this study is the low number of genotype combinations used for the BBAC hybrids resulting from one *B. juncea* and two *B. carinata* genotypes (J1C1 and J1C2). This is important because genotype has been shown to affect the chromosome pairing behavior of *Brassica* interspecific hybrids (Jenczewski *et al*., 2003; Mason *et al*., 2010). It is therefore possible that if the numbers of genotypes are increased, then we might observe some differences in the consistent fertility increase and meiotic stability we see across generations. On the other hand, the results from the AABC and CCAB show that the effect of genotype might also not be significant. In the AABC and CCAB hybrid even with the higher number of genotype combinations, these hybrids still portrayed very poor fertility within the different genotype combinations. It would however be worthwhile increasing the genotype combination in the BBAC hybrids by increasing the number of *B. juncea* and the number of *B. carinata* genotypes. From this we can access the effect of genotype on the fertility and meiotic pairing of BBAC hybrids.

The second limitation of this study is about the experimental design. Multiple locations which were not consistent across generations were used and the selective pressure applied across the different generations was not uniform. Additionally at the end, the fertility was not tested in the final generations across multiple sites. While efficient meiosis is critical for fertility the

process can indeed be perturbed by environmental factors leading to deleterious outcomes such as reduced fertility or aneuploidy (Hassold and Hunt, 2001; Inoue and Lupski, 2003; Bomblies *et al*., 2015). The high fertility of the BBAC hybrids or the low fertility of the AABC and CCAB hybrids could also have been influenced by environmental factors. Adding replications and varying the location could help us draw stronger conclusions on the pairing behavior, stability and fertility of these hybrids. Additionally, the selective pressure applied across S_1 to S_3 was not uniform. The variable selective pressures applied to the different generations (three parent plants in the S_1 generation, two in the S_2, three in the S_3 and four in the S_4, S_5 and S_6) could have also introduced some biases to the study. Applying a constant selective pressure could maybe produce a different outcome. Another option would have been to separate the materials into two groups: the highest fertility plants and the lowest fertility plants and analyze how these groups respond separately to selective pressure. If the plant with high fertility can improve in stability and fertility, then maybe low fertility plants can also improve their fertility and stability. Applying the selective pressure just in a single direction may leads to possible valuable genetic resource being discarded. Another limitation of this study is the absence of GISH results to show how the chromosomes are pairing with each other. In hybrids, balanced aneuploidy can occur where the loss of one chromosome is replaced by its homoeologues (Xiong *et al*., 2011; Zhang *et al.*, 2013a). Some of such changes might not have been picked up by the SNP data. Additionally, some intergenomic recombination might have occurred between the A and C genome with some of the B genome chromosomes which might have been missed by the SNP data analysis.

However, notwithstanding the above limitations, the many generations of selection, and given that not only a single genotype per species was used but with consistent results, gives credibility and reliability to the results and conclusions drawn from this study. Additionally, the SNP genotyping data gave a good coverage of chromosome inheritance and enabled us to have a deeper insight into the structural changes occurring in the chromosomes.

5.4 Conclusions and future perspective

Interspecific hybridization presents an enormous opportunity to not only create useful genetic variation but to also transfer variation between crop species. However, in polyploids meiotic chromosome pairing can be a challenge. There exists limited understanding of the cytological changes which take place when *Brassica* trigenomic allotetraploids are formed. In particular, it is unknown whether selection across several generations which represents a form of evolutionary selection and adaptation can eliminate the instabilities common to newly formed polyploidy species and hence give rise to new stable species or karyotpes. This thesis aimed to answer the question: if we create trigenomic *Brassica* hybrids and self-pollinate these hybrids for several generations while selecting for fertility can we recover stable fertile hybrids after several generations? Can the chromosomes restructure and pair correctly?

Brassica AABC, BBAC and CCAB trigenomic hybrids were studied with a particular focus on BBAC hybrids. First we analyzed the fertility and chromosome pairing behavior of all hybrid types in the F_1 generation and in the S_1 generation using the *Brassica* Illumina Infinium SNP data. The fertility of the hybrids varied significantly between the hybrid types. CCAB hybrids had the lowest fertility followed by AABC and then BBAC. Except for BBAC, the fertility of the other hybrid types did not show any significant increase from F_1 to S_1. Instead CCAB hybrids became infertile by the S_1 generation. Analysis of S_1 hybrids showed the A and B genome in the CCAB hybrids were mostly unpaired with a high loss of B-genome chromosomes compared to the A. With the BBAC hybrids, a high number of homoeologous exchanges were observed between the A and C genomes with the B genome showing no homologous pairing. For the AABC hybrids, a few homoeologous exchanges could be observed between the C and the A genome but not with the B genome. There was also loss of B genome chromosomes. The results showed that the fertility and chromosome pairing of trigenomic *Brassica* allotetraploids depends on the genomic composition of the hybrid type. This will have implications when trying to transfer useful genetic information from one *Brassica* species to another, especially between allotetraploids. From these hybrids, depending on the nature of the homoeologous exchange

and the chromosomes involved, homoeologous pairing can either be detrimental or beneficial to the fertility and stability of these hybrids. Loss of chromosomes can also have a negative effect on fertility compared to homoeologous pairing. This can be seen with the CCAB where loss of B chromosomes was not favorable to the fertility of the hybrids.

In the second study using the BBAC hybrids we establish that new stable and fertile karyotpes can be formed in later generation interspecific hybrids following selection for fertility. The haploid A and C genomes were able to pair, recombine, and restructure, and behaved like homologues thereby leading to increased meiotic stability and fertility as the B genome maintained pairing fertility. The case of the A/C genome in the BBAC lines can be likened to the case of homoploid hybrid speciation where two species come together without a change in ploidy number. This can lead to the establishment of new crop species.

Generating new hybrid types can have significant effects on plant breeding by generating new genetic variation. Besides creating new genetic diversity, assessing the usefulness of this newly created genetic variation becomes important. Therefore, testing these hybrids for important traits such as disease resistance, drought tolerance, and other biotic and abiotic factors will lend usefulness to these hybrids. These hybrids can also be backcrossed to other established cultivars or species and tested for important agronomic traits. Assessing the seed quality characteristics of these hybrid types would also add to the usefulness of these hybrids. The possibility of increasing crossover frequency has attracted considerable interest because of the obvious practical applications in traditional breeding and the genetic studies. Recent results suggest that manipulating karyotype composition could be a new way to increase crossover frequency in plants (Leflon *et al*., 2010; Suay *et al.*, 2014; Pelé e*t al*., 2018). Therefore, analyzing the crossover frequency in these hybrids can shed light on how the genome structure affects recombination of these hybrids. Accessing recombination both between the B genome and the haploid A and C genomes will add to the usefulness of these hybrids. Through this information we can be able to create more genetic diversity. Talking about assessing recombination, molecular cytogenetics using BAC-FISH becomes important. By using this technique on these hybrids, we can have more insight into the pairing behavior of these hybrids: information which we may not be able to know using just SNP analysis and classical cytogenetics. Another possible future direction for these hybrids is to self-pollinate and see if the increase continues and if they maintain their stability and fertility or is possible that this stability can break down at a certain point.

6.0 References

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7.1 Appendices

Appendix I: Supplementary Information chapter 3

New Phytologist Supporting Information

Article title: Stable, fertile lines produced by hybridisation between allotetraploids *Brassica juncea* (AABB) and *B. carinata* (BBCC) have merged the A and C genomes.

Authors: Elvis Katche[1], Roman Gaebelein[1], Zurianti Idris[2], Paula Vasquez-Teuber[1,2,4], Yu-tzuLo[2], David Nugent[2], Jacqueline Batley[3] and Annaliese S. Mason[1,2,5*]

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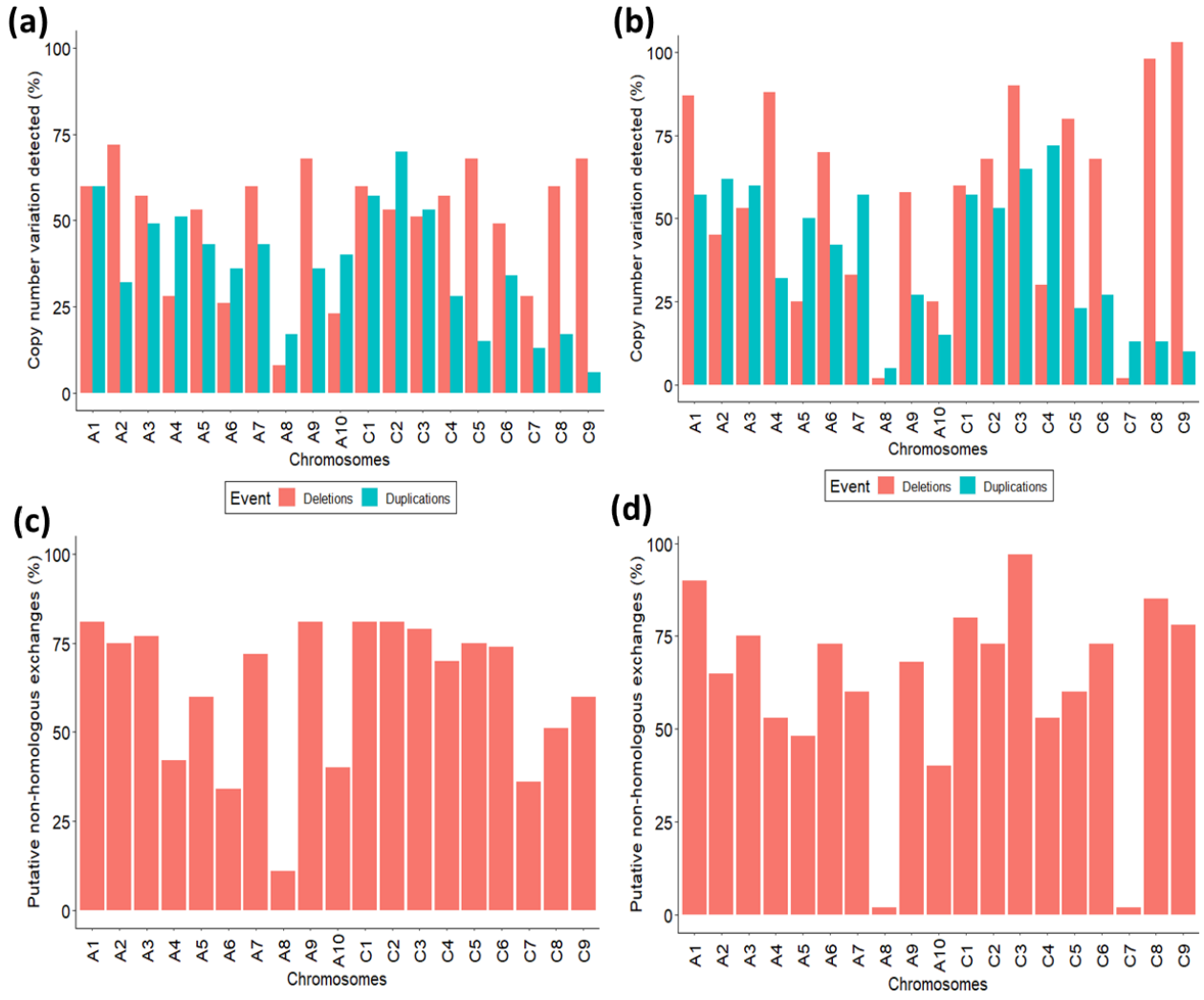
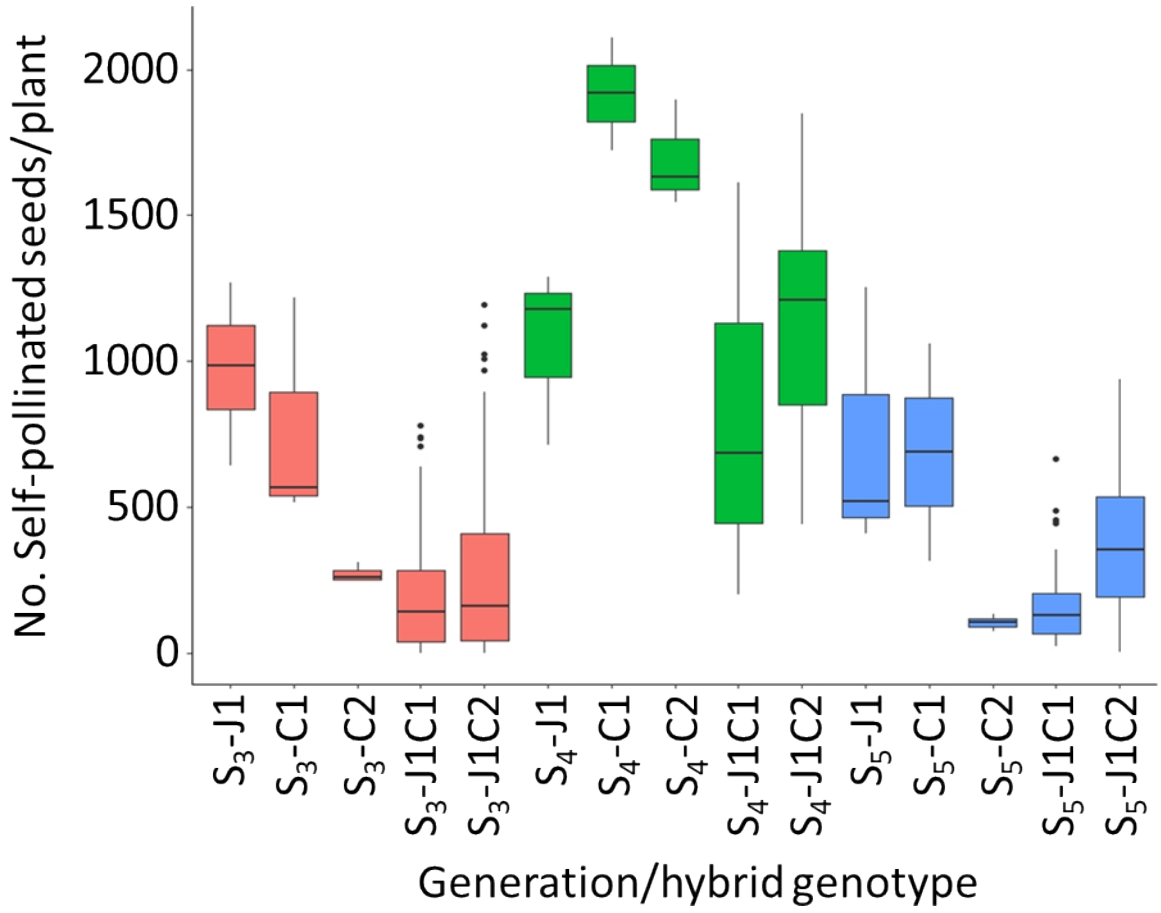
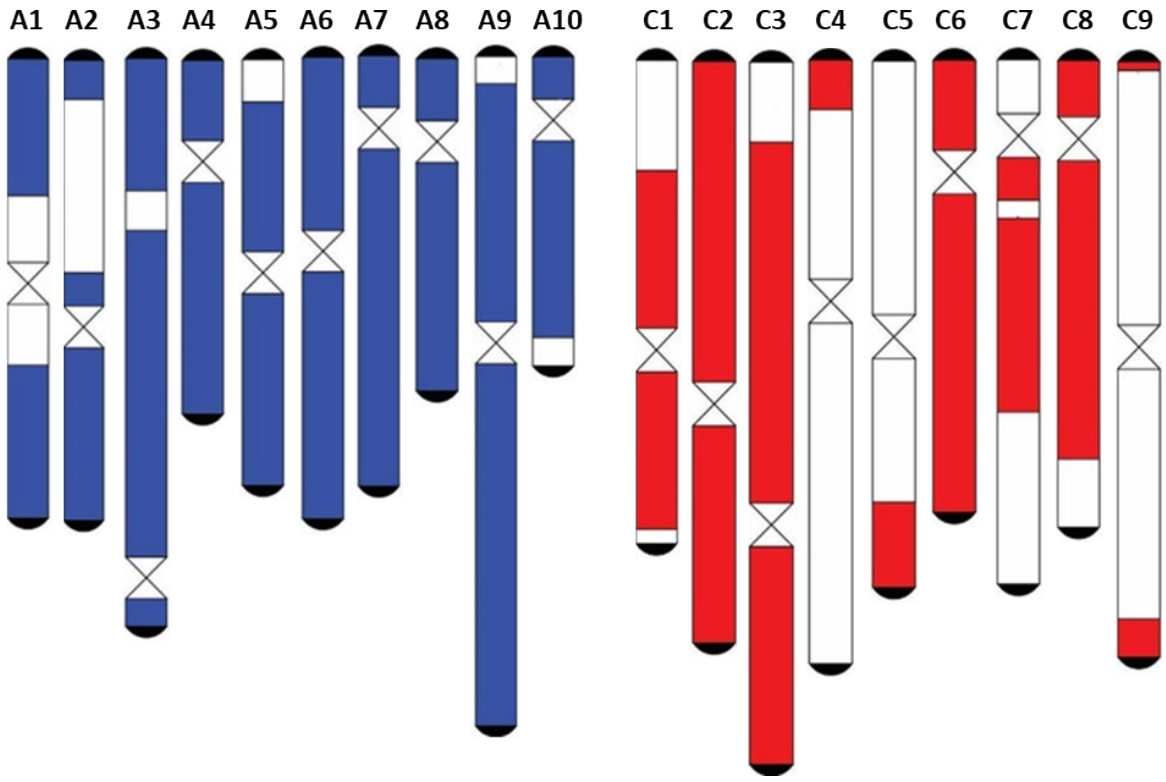
7.1 Supplementary figures



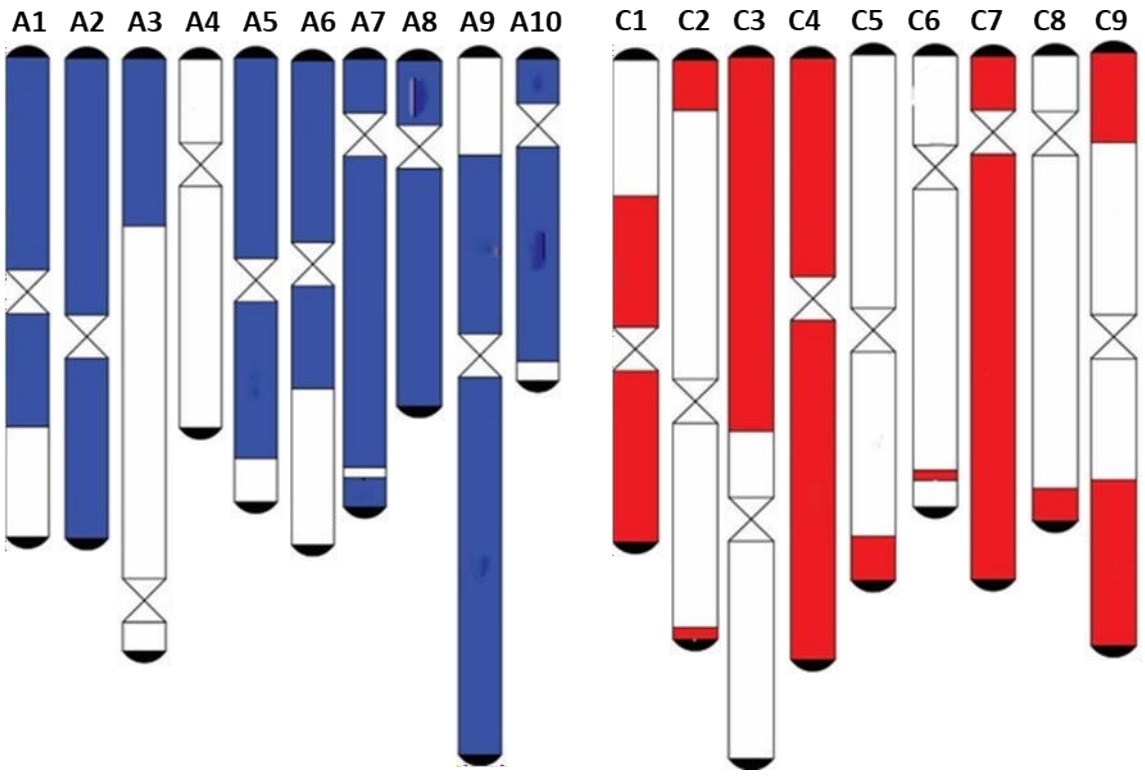
Supporting information Figure S1. Genetic changes in *Brassica juncea* × *Brassica carinata* self-pollinated S_2 interspecific hybrids as detected from Illumina Infinium *Brassica* 60K SNP array data: **a)** Percentage of deletions/duplications (loss or gain of a whole or part of a chromosome) in the J1C1 population **b)** Percentage deletions/duplications in the J1C2 population for the different A and C chromosomes **c)** percentage of individuals with recombinant chromosomes resulting from non-homologous recombination events for each A- and C-genome chromosome in the J1C1 S_2 population and **d)** individuals with recombinant chromosomes resulting from non-homologous recombination events for each A- and C-genome chromosome in the J1C2 S_2 population.



Supporting information Figure S2. Fertility of *Brassica juncea* × *B. carinata* interspecific hybrids in S_3, S_4 and S_5 generations of self-pollination with selection for fertility relative to their parent genotypes (*B. juncea* "J1", *B. carinata* "C1" and *B. carinata* "C2"). Where red is the S_3, green S_4 and blue S_5 generation.



Supporting information Figure S3. A- and C- genome presence and absence based on SNP marker genotyping in hybrids derived from the cross *B. juncea* × *B. carinata* (J1C1 genotype) following two generations of self-pollination (BBAC S_2) and selection for fertility. Blue represents presence of the A genome, white represents loss of whole chromosomes or chromosome segments, and red represents presence of the C genome.



Supporting information Figure S4. A- and C- genome presence and absence based on SNP marker genotyping in hybrids derived from the cross *B. juncea* × *B. carinata* (J1C2 genotype) following two generations of self-pollination (BBAC S_2) and selection for fertility. Blue represents presence of the A genome, white represents loss of whole chromosomes or chromosome segments, and red represents presence of the C genome.

7.2 Supplementary tables

Supporting information Table S1. Meiotic chromosome pairing configurations of hybrids produced from the cross *Brassica juncea* × *Brassica carinata* followed by six generations of self-pollination (BBAC S_6) for two genotypes "J1C1" and "J1C2" produced from the same *B. juncea* but different *B. carinata* along with parent controls. I, II and III refer to univalents (unpaired chromosomes), bivalents (chromosome pairs) and trivalents (three associated chromosomes) respectively. PMC = pollen mother cell

Progeny set	Plant	Chromosome number	No. of PMCs counted	No. of I	No. of II	No. of III	Av. I / PMC	Av. II / PMC	Av. III/ PMC
J1C1-S6_1	1	36	40	16	709	2	0.2	17.7	0.1
J1C1-S6_1	2	36	20	4	358	0	0.1	17.9	0
J1C1-S6_2	1	36	40	17	354	1	0.2	17.8	0
J1C1-S6_2	2	36	40	14	713	0	0.2	17.8	0
J1C1-S6_3	1	36	40	10	715	0	0.1	17.9	0
J1C1-S6_3	2	36	40	3	717	0	0.1	17.9	0
J1C1-S6_4	1	35	40	50	675	0	0.6	16.9	0
J1C1-S6_4	2	36	40	14	711	1	0.2	17.8	0
J1C2-S6_1	1	36	40	12	711	2	0.1	17.8	0.1
J1C2-S6_1	2	36	20	5	356	1	0.1	17.8	0.1
J1C2-S6_2	NA	NA	NA	NA	NA	NA	NA	NA	NA
J1C2-S6_2	NA	NA	NA	NA	NA	NA	NA	NA	NA
J1C2-S6_3	1	36	20	28	335	1	0.7	17.4	0.1
J1C2-S6_3	2	36	20	18	351	0	0.5	17.5	0
J1C2-S6_4	1	36	20	6	357	0	0.2	17.9	0
J1C2-S6_4	2	36	20	6	357	0	0.2	17.9	0
J1	1	36	20	0	360	0	0	18	0
C1	1	34	20	0	340	0	0	17	0
C2	1	34	20	0	340	0	0	17	0

Supporting information Table S2. Putative centromere locations for the *Brassica* A - and C - genomes on the Darmor-*bzh* v8.1 reference genome estimated using half-tetrad analysis of mapping populations of *Brassica juncea* × *Brassica napus* (AABC) and *Brassica carinata* × *Brassica napus* (CCAB) hybrids genotyped using the Illumina Infinium *Brassica* 60K array (see Mason et al. 2016 (Mason et al., 2016); data from this paper was remapped to the new genome assembly).

Chromosome	Start position (Mbp)	Flanking SNP (start)	End position (bp)	Flanking SNP (end)
A01	15.2	Bn-scaff_20478_1-p135109	15.3	Bn-A01-p16131102
A02	18.3	Bn-A02-p17012938	22.6	Bn-A02-p18949958
A03	37.2	Bn-scaff_16110_1-p1049197	39.4	Bn-Scaffold000372-p15947
A04	6.0	Bn-A04-p4978780	6.9	Bn-Scaffold000104-p158234
A05	14.3	Bn-A05-p12056452	14.4	Bn-A05-p14313107
A06	13.0	Bn-A06-p15375170	13.6	Bn-A06-p12363034
A07	3.8	Bn-A07-p1482858	6.1	Bn-A07-p3287486
A08	4.6	Bn-A08-p4538107	5.9	Bn-A08-p6185551
A09	19.8	Bn-A03-p24124318	20.2	Bn-A09-p19181075
A10	3.2	Bn-A10-p515442	5.2	Bn-A07-p21689339
C01	29.2	Bn-scaff_15906_1-p593282	29.6	Bn-scaff_15906_1-p190868
C02	34.9	Bn-scaff_17067_1-p111399	35.0	Bn-scaff_17067_1-p175455
C03	48.1	Bn-scaff_21330_1-p291816	48.2	Bn-scaff_18406_1-p157903
C04	23.9	Bn-scaff_19575_1-p956958	25.5	Bn-scaff_18562_1-p37458
C05	27.7	Bn-A05-p11840240	28.4	Bn-scaff_22461_1-p485
C06	9.8	Bn-scaff_17454_1-p87022	10.0	Bn-scaff_17454_1-p254299
C07	5.9	Bn-scaff_17461_1-p626637	6.0	Bn-scaff_16721_1-p2190132
C08	6.3	Bn-scaff_16158_1-p377774	6.6	Bn-scaff_16962_1-p473848
C09	28.7	Bn-scaff_19661_1-p165828	29.0	Bn-scaff_16297_1-p165841

Supporting information Table S3: Meiotic pairing of F_1, S_4, S_5 and S_6 generation hybrid plants. Forty Pollen mother cells (PMCs) were counted for each generation to obtain the average number of univalents (I), bivalents (II), trivalents (III) and tetravalent (IV).

Generation	Chromosomes number	PMCs counted	I	II	III	IV
F_1	35	40	3.1	11.7	2.6	0.2
S_4	34	40	0.7	16	0.2	0
S_5	36	40	0.3	17.16	0.14	0
S_6	36	40	0.2	17.8	0.03	0

Supporting Information Table S4: Seed information for self-pollinated *B. juncea* × *B. carinata* hybrids from S_1 - S_6 showing the number of plants which were grown in each generation and under which condition and the number SNP genotyped. It also shows the number of plants which were removed from analysis.

	J1C1				J1C2			
Geneation	Green house grown	Field grown	Genotyped	Contaminated/ Failed samples	Green house grown	Field grown	Genotyped	Contaminated/ Failed sample
S1	20	113	20	0	24	107	20	4
S2	44	0	44	5	62	0	62	2
S3	237	0	88	0	160	0	92	0
S4	50	0	0	0	50	0	0	0
S5	48	0	48	0	48	0	48	0
S6	48	0	48	0	48	0	48	0

7.3 Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in this dissertation. I have indicated in the text where I used text from already published sources either word for word or in substance and where I have made statements based on oral information given to me as described in the dissertation. I have followed the principle of good scientific practice as defined in the "Statutes of Justus Liebig University Gießen for Safeguarding of Good Scientific Practice".

Giessen, Monday 9th October 2023 **Elvis Katche**

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