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Summary

Rapeseed (*Brassica napus*, AACC) is a young allotetraploid species formed by the hybridization of *Brassica rapa* (AA) and *Brassica oleracea* (CC). The genetic diversity of rapeseed is limited as a result of few hybridization events between diploid progenitor genotypes as well as intense breeding selection for oil quality traits. One possible way to increase the genetic diversity is by generating resynthesized *B. napus* lines via interspecific hybridization of the diploid progenitor species *B. rapa* and *B. oleracea*. However, resynthesized *B. napus* lines are often meiotically unstable and infertile, unlike natural *B. napus*, which is both fertile and stable. This prevents their long-term maintenance and direct use in breeding programs. One hypothesis is that meiotic stability in established *B. napus* may have arisen through the inheritance of specific alleles from their diploid progenitors. This hypothesis was tested by assessing copy number variation and fertility of 41 early generation (S_1) resynthesized lines with SNP genotyping information produced from crosses between eight *B. rapa* and eight *B. oleracea* genotypes. Subsequently, eight *B. rapa* and five *B. oleracea* parent accessions were resequenced and nineteen resynthesized *B. napus* lines were analysed for allelic variation in a list of meiosis gene homologs. A second group of resynthesized *B. napus* material; a large diverse set of 140 lines including early (S_1) and advanced generation resynthesized genotypes produced by crosses between *B. rapa* and *B. oleracea* as well as between *B. rapa* and wild C genome species (*B. incana*, *B. hilarionis*, *B. montana*, *B. bourgeaui*, *B. villosa*, and *B. cretica*) was assessed for purity (homozygosity), fertility, and genome stability. Two major results were obtained in this thesis. Firstly, the identification of 13 putative meiosis candidate genes with presence of putatively harmful mutations and which significantly affect the frequency of copy number. Second is the observation of 8 genomically stable resynthesized *B. napus* lines. The results obtained in this thesis suggests that meiotic stability in established *B. napus* arose via selection of specific alleles inherited from its diploid progenitor species. This information is useful to breeders who aim to use resynthesize lines as direct breeding materials or for the introgression of useful traits into elite *B. napus* cultivars. The observation of a few stable and fertile resynthesized lines shows that it is possible to maintain resynthesized *B. napus* as useful germplasm resource for research and breeding.

Zusammenfassung

Raps (*Brassica napus*, AACC) ist eine junge allotetraploide Art, die aus der Hybridisierung von *Brassica rapa* (AA) und *Brassica oleracea* (CC) hervorgegangen ist. Die genetische Vielfalt des Rapses ist aufgrund der wenigen Hybridisierungsereignisse zwischen den diploiden Vorläufergenotypen sowie der intensiven züchterischen Selektion auf Ölqualitätsmerkmale begrenzt. Eine Möglichkeit, die genetische Vielfalt zu erhöhen, besteht in der Erzeugung von resynthetisierten *B. napus*-Linien durch interspezifische Hybridisierung der diploiden Vorläuferarten *B. rapa* und *B. oleracea*. Resynthetisierten *B. napus*-Linien sind jedoch häufig meiotisch instabil und unfruchtbar, im Gegensatz zur Natürlichen *B. napus*, die sowohl fruchtbar als auch stabil ist. Dies verhindert ihre langfristige Erhaltung und Direkte Verwendung in Zuchtprogrammen. Eine Hypothese besagt, dass die meiotische Stabilität in etablierte *B. napus* durch die Vererbung spezifischer Allele aus ihren diploiden Vorfahren entstanden sein könnte. Diese Hypothese wurde getestet, indem die Kopienzahlvariation und die Fruchtbarkeit von 41 resynthetisierten Linien der ersten Generation (S_1) mit SNP- Genotypisierungsinformationen aus Kreuzungen zwischen acht *B. rapa*- und acht *B. oleracea*- Genotypen bewertet wurden. Anschließen wurden acht *B. rapa*- und fünf *B. oleracea*- Elternakzessionen resequentiert und neunzehn resynthetisierten *B. napus*-Linien auf allelische Variation in einer Liste von Meiosegen-Homologie analysiert. Eine zweite Grüppe von resynthetisiertem *B. napus*-Material eine Große Vielfalt von 140 Linien darunter frühe (S_1) und fortgeschrittene Generationen von resynthetisierten Genotypen, die durch Kreuzungen zwischen *B. rapa* und *B. oleracea* sowie zwischen *B. rapa* und wilden C-Genom-Arten (*B. incana*, *B. hilarionis*, *B. montana*, *B. bourgeaui*, *B. villosa*, und *B. cretica*) erzeugt wurden, wurde auf Reinheit (Homozygotie), Fruchtbarkeit und Genomstabilität untersucht. In dieser Arbeit wurde zwei wichtige Ergebnisse erzielt. Erstens die Identifizierung von 13 mutmaßlichen Meiose-Kandidatengenen, in denen mutmaßlich schädliche Mutationen vorhanden sind und die die Haüfigkeit der Kopienzahl erheblich Beeinflussen. Zweitens die Beobachtung von acht genomisch stabilien resynthetisierten *B. napus*-Linien. Die in dieser Arbeit erzielten Ergebnisse lassen vermuten, dass die meiotische Stabilität in der etablierten *B. napus* durch die Selektion spezifischer Allele, die von den Diploiden Vorgängerspezies geerbt wurden, entstanden ist. Die Beobachtung einniger stabiler und fruchtbarer resynthetisierter Linien zeigt, dass es möglich ist, resynthetisierte *B. napus* als nützliche Keimplasma-Ressource für Forschung und Züchtung zu erhalten.

1.0 Introduction

1.1 Origin and genetic diversity of rapeseed

The *Brassica* genus is one of 51 genera in the tribe Brassiceae belonging to the crucifer family (Brassicaceae) and is the most economically important genera within this tribe (Rakow 2004). It is an interesting model for allopolyploid formation in agricultural crops, as six agriculturally significant species share a genomic relationship (U 1935).The predecessors of the diploid species *B. rapa* (A genome, 2n = 20, Chinese cabbage and turnip), *B.nigra* (B genome, 2n = 16, black mustard) and *B. oleracea* (C genome, 2n = 18, cabbage, cauliflower, broccoli) are hypothesized to have given rise to the allotetraploid species *B. juncea* (A and B genome 2n= 36, leaf mustard, Indian mustard), *B. napus* (A and C genome, 2n = 38, oilseed rape, canola) and *B. carinata* (B and C genome, 2n = 34, Ethiopian mustard) through pairwise hybridization (Morinaga 1934; U 1935). *B. napus* (genome $A_nA_nC_nC_n$) was spontaneously formed by recent allopolyploidy between ancestors of *B. oleracea* (Mediterranean cabbage, genome C_oC_o) and *B. rapa* (Asian cabbage or turnip, genome A_rA_r) in the last 7500 years (U 1935), with at least two hybridization events (Allender and King 2010; Chalhoub et al. 2014).

However, no wild *Brassica napus* is known to exist, making it challenging to determine its precise origin. Recent studies have shown that the *Brassica napus* A-subgenome may have evolved from *B. rapa* spp. *rapa*, which is the ancestor of European turnip (Yang et al. 2016; Lu et al. 2019). On the other hand, the C- subgenome has been shown to have a more complex origin than the A-subgenome, possibly evolving from either the wild C genome *Brassica* species *B. montana* (Becker 1992) or the common ancestor of four lineages of *B. oleracea* comprising Kohlrabi (*B. oleracea var. gongylodes*), cauliflower (*B. oleracea* var. *botrytis*), broccoli (*B. oleracea* var. *italica*), and Chinese kale (*B. oleracea* var. *alboglabra*) (Lu et al. 2019). This may support the hypothesis that the original hybridization event that resulted in *B. napus* may have occurred on more than one occasion and involved different maternal genotypes (Allender and King 2010).

Brassica napus, also known as rapeseed or canola, is one of the most important oilseed crops produced globally. Vegetable (fodder rape and kale) and tuberous forms (Swede and rutabaga) of *B. napus* are used for human consumption and animal fodder (Allender and

King 2010). Due to intensive breeding effort to produce “double low” (low erucic acid and glucosinolate contents in seeds), the global significance of rapeseed has increased immensely over the last decades.

However, this breeding effort has further eroded the genetic diversity of rapeseed, which was already limited by the few initial hybridization events between the diploid *B. rapa* and *B. oleracea* parents that produce *B. napus* (Delourme et al. 2013) as well as the limited history of cultivation and domestication (Prakash et al. 2012). Therefore, expanding the genetic diversity of rapeseed is imperative in order to increase genetic variation in the *B. napus* breeding pool. Over the years, several methods have been studied such as physical and chemical mutagenesis, hybridization as well as genetic engineering in order to improve the current rapeseed gene pool (Hu et al. 2021). However, hybridization especially interspecific hybridization is potentially an efficient way to expand genetic diversity in rapeseed.

1.2 Agronomic potential of resynthesized *Brassica napus* in rapeseed breeding

Resynthesized *Brassica napus* lines are potentially of great interest in hybrid breeding because strong breeding selection for agronomically useful traits by breeders has eroded the gene pools of many *B. napus* cultivars (Girke et al. 2012a; Jesske et al. 2013; Szała et al. 2016). One strategy to overcome this problem is to exploit the diploid progenitors of crop species and/or their wild crop relatives as sources of novel favourable alleles for the improvement of current *B. napus* breeding lines (Udall et al. 2004) as well as for the broadening of genetic diversity (Girke et al. 2012a; Rahman et al. 2015; Wu et al. 2015). Becker et al. (1999) suggested the possibility of using resynthesized lines to produce a genetically diverse winter oilseed rape gene pool that can be used in hybrid breeding. One way to do this is to create semi- resynthesized *B. napus* lines produced by crossing rapeseed cultivars with resynthesized lines in order to improve the agronomic potential of the current rapeseed gene pool. Using interspecific hybridization, semi-resynthesized lines with improved agronomic characters compared to pure resynthesized lines have been produced. Girke et al. (2012b) reported higher yields in some semi- resynthesized genotypes compared to *B. napus* cultivars, although low yield was still a major problem. Jesske et al. (2013) investigated the use of 29 resynthesized lines derived from wild C genome species (*B. incana, B. hilarionis, B. montana, B. Bourgeaui, B. villosa* and *B. cretica*) to broaden the genetic diversity and improve heterosis in *B. napus* cultivars.

Resynthesized lines from wild *Brassica* species produced higher yields when crossed with established *B. napus* varieties (Jesske et al. 2013).

Resynthesized *B. napus* has also been studied for the introgression of several agronomically useful traits such as disease resistance (Leflon et al. 2007; Obermeier et al. 2013; Niemann et al. 2017; Kawasaki et al. 2021), drought resistance/tolerance (Jiang et al. 2019), early flowering (Schranz and Osborn 2000; Rahman et al. 2011), pod shatter resistance (Morgan et al. 1998; Summers et al. 2003), and insect resistance (Eickermann and Ulber 2011; Schaefer et al. 2017). However, resynthesized lines produced so far still show poor agronomic performance as well as other undesirable seed quality traits such as high erucic acid and glucosinolate content in seeds, which complicates the direct introduction of resynthesized lines into hybrid breeding programs (Girke et al. 2012a, b; Jesske et al. 2013; Szała et al. 2019). Girke et al. (2012a) generated resynthesized lines for several generations and subsequently preselected for better agronomic performance and seed set. Others have improved the agronomic performance of resynthesized oilseed rape by producing semi-resynthesized lines from crosses between resynthesized *B. napus* lines and established double-low oilseed rape, leading to the production of double low quality semi-resynthesized lines that can be used in oilseed rape breeding (Szała et al. 2016, 2019). Semi-resynthesized lines with increased pod shatter resistance compared to *B. napus* cultivar have also been produced (Summers et al. 2003). Resynthesized *B. napus* genotypes with early flowering, as well as short life cycle duration traits have been successfully produced by crossing early varieties of *B. rapa* and *B. oleracea* (Akbar 1987; 1990; Karim et al. 2014; Das et al. 2022). Schaefer et al. (2017) detected one resynthesized line with higher resistance to the insect; *Ceutorhynchus napi* Gyll. compared to *B. napus* cultivars, and to other resynthesized lines analysed. Most *B. napus* cultivars are susceptible to major diseases that affect rapeseed, and sources of resistance are either lacking or limited in the current rapeseed gene pool. Breeding resistant *B. napus* cultivars is however necessary for long term management of any disease in rapeseed (Rahman et al. 2014). Enhanced resistance to several major diseases of rapeseed such as blackleg (Crouch et al. 1994; Leflon et al. 2007; Yu et al. 2012), *Verticillium* wilt (Rygulla et al. 2007; Eynck et al. 2009; Obermeier et al. 2013), *Sclerotinia* stem rot (Mei et al. 2011, 2015; Ding et al. 2013), and clubroot disease (Kawasaki et al. 2021) have been observed in resynthesized lines compared to elite *B. napus* cultivars. Many resistances have been sourced from either the

diploid progenitors *B. rapa* and *B. oleracea* or wild *Brassica* species, and successfully transferred into elite *B. napus* (Yu et al. 2012; Mei et al. 2015; Kawasaki et al. 2021). Resynthesized rapeseed is an indispensable genetic resource for the expansion of genetic diversity in rapeseed as well as for the introgression of desirable traits into elite rapeseed cultivar (Katche and Mason 2023).

1.3 Polyploidy and interspecific hybridization

Polyploidy is the heritable condition in which organisms or cells contain more than two sets of chromosomes. Polyploidy is grouped into allopolyploids, in which two or more genomes from different species are hybridized to form a new polyploid, and autopolyploids, where more than one homologous copy of the same chromosome is present in the resulting polyploid (Bomblies 2023). Polyploidy is prevalent in plants, especially in angiosperms (Jiao et al. 2011; Pelé et al. 2018), and has been attributed to the success of many plant species. Polyploids show more evolutionary advantage over their diploid parents in terms of heterosis, which allows offspring to agronomically perform better than their diploid parents (Birchler et al. 2010). Secondly, gene redundancy, which is the presence of multiple gene copies performing the same function, may help to mask the deleterious effect of gene mutations, thereby preventing loss of fitness (Gu et al. 2003). Many of our crop plants are polyploids, and these advantages make polyploidy a promising tool for crop improvement (Udall and Wendel 2006; May et al. 2023) as well as for understanding how meiosis evolves in polyploid plants (Bomblies 2023).

The prospect of recreating speciation events over shallow evolutionary time via interspecific hybridization offers the potential advantage of studying meiosis and genome evolution in order to understand how two genomes come together to form a species (Katche and Mason 2023). Interspecific hybridization involves the crossing of two different species belonging to the same genus to form a hybrid. The use of interspecific hybridization to reproduce *B. napus* (AACC, 2n = 38) is potentially of great interest in breeding and in the study of genome evolution because it can be readily synthesized from crosses between two highly divergent parental species *B. rapa* (AA, 2n = 20) and *B. oleracea* (CC, 2n = 18). The most common method to resynthesize *B. napus* is by deliberate sexual crosses via hand pollination between progenitor species *B. rapa* and *B.*

oleracea to produce the haploid F_1 hybrid, followed by chromosome doubling using colchicine to derive tetraploid resynthesized *B. napus* lines (Seyis et al. 2003; Abel et al. 2005; Rygulla et al. 2007; Girke et al. 2012a; Malek et al. 2012; Karim et al. 2014). Japanese scientists probably made the first attempt to produce resynthesized *B. napus* with the earliest reports possibly as far back as 1935 by the Japanese scientist U Nagaharu. U (1935) crossed nine different cultivars each of *B. rapa* and *B. oleracea* but produced only one spontaneous allotetraploid *B. napus* F_1 hybrid. Early attempts by scientists to produce resynthesized *B. napus* lines via interspecific hybridization using hand-pollination produced only a limited number of allotetraploid F_1 hybrids (U 1935; Hoffmann and Peters, 1958; Olsson 1960). Most hybrids produced by interspecific crosses involving *B. oleracea* as the maternal parent were largely sterile (U 1935; Nishi et al. 1959; Olsson 1960; Hosoda et al. 1963) compared to *B. rapa* × *B. oleracea* reciprocal crosses, with a few exceptions (Hoffmann and Peters 1958; Takeshita et al. 1980) as reviewed by (Katche and Mason 2023), although only heterozygous parent lines were crossed in these early studies. In the twentieth century, the focus of many studies was mostly targeted towards producing interspecific *B. napus* hybrids, and analysing their meiotic stability. However, recent studies have aimed to produce resynthesized *B. napus* lines by crossing homozygous double haploid or inbred *B. rapa* and *B. oleracea* parent lines in order to increase genetic variation (Girke et al. 2012a; Jesske et al. 2013; Das et al. 2022) introgress desirable agronomic traits (Jiang et al. 2013; Ding et al. 2019) as well as for evolutionary studies to understand how meiosis was initially stabilized in established *B. napus* (Rousseau-Gueutin et al. 2017; Ferreira de Carvalho et al. 2021; Higgins et al. 2021; Xiong et al. 2021). Genome instability and reduced fertility as a result of abnormal meiosis still remains a major challenge in neopolyploids (reviewed by Pelé et al. 2018), including resynthesized *B. napus* lines. Addressing this problem continues to be the focus of many studies in the present century.

1.4 Meiotic instability of resynthesized lines

Meiosis is a reductional cell division which takes place in sexually reproducing organisms giving rise to offspring with half the number of chromosomes of their parents. Meiotic programs take place in two phases; a single phase of DNA replication followed by a two-step division. Meiotic recombination takes place in the first step, and is important for ensuring genome stability and fertility, as well as creating new genetic

diversity (Pelé et al. 2018; Gonzalo 2022). However, in newly formed allopolyploids, homoeologous pairing occurs between chromosomes from ancestrally related subgenomes with high sequence similarity during meiosis (Mason and Wendel 2020). This complexity in allopolyploid meiosis compromises genome stability and hence fertility.

Interestingly, resynthesized *B. napus* has been shown to be meiotically unstable, unlike natural *Brassica napus* (Jenczewski et al. 2003; Szadkowski et al. 2010; Xiong et al. 2011; Nicolas et al. 2012; Grandont et al. 2014; Rousseau-Gueutin et al. 2017; Ferreira de Carvalho et al. 2021). Although low frequencies of homoeologous pairing are still observed in natural *Brassica napus* (Chalhoub et al. 2014; Xiong et al. 2021), resynthesized *B. napus* lines show different levelsof meiotic abberations, depending on the parent genotype or combinations used for their hybridization (Szadkowski et al. 2010), which may influence the extent of homoeologous recombination in these hybrids (Nicolas et al. 2007; Grandont et al. 2014). Szadkowski et al. (2010) analyzed meiosis in three different early generation (S_0) resynthesized *B. napus* lines at metaphase 1, and observed frequent A-C bivalents and multivalents in 30 – 47.5 % of pollen mother cells. These meiotic abnormalities were suggested to be more likely transmitted to subsequent generations (Szadkowski et al. 2010). Xiong et al. (2011) analyzed both early (S_0) and late (S_6) generation resynthesized *B. napus* and observed a 3-fold increase in aneuploidy (frequency of extra or missing chromosomes) over subsequent generations. Meiosis in four natural *B. napus* cultivars, as well as one resynthesized *B. napus* line in both S_0 and S_{11} generation were investigated: a high frequency of homoeologous pairing was observed in highly syntenic regions of chromosomes in the resynthesized lines but was rare in natural *B. napus*, as indicated by the low number of tetravalents observed (Xiong et al. 2021). This suggests that established *B. napus* possibly achieved meiotic stability by suppression of homoeologous pairing via one or more pairing control genes (Jenczewski et al. 2003; Liu et al. 2006; Higgins et al. 2021).

Recent reports suggest several possible routes to achieving meiotic stabilization in *B. napus*, and in other neopolyploids (Gonzalo 2022; Bomblies 2023; Katche and Mason 2023). Firstly, inheritance of pre-existing meiotic allelic variants from diploid progenitors has been hypothesized as one means to stabilize meiosis (Mason and Wendel 2020; Gonzalo 2022). Results obtained from a few previous studies seem to support this idea. Samans et al. (2017) detected de novo allelic variants from analysing natural and

synthetic *B. napus* which could be potential drivers for meiotic stabilization, as reviewed by (Katche and Mason 2023). Some early generation resynthesized genotypes show more stable meiosis compared to others, possibly influenced by allelic variants from their diploid progenitors (Szadkowski et al. 2010). Secondly, de novo mutations occurring immediately after whole genome duplication may possibly restore meiotic stability in neopolyploids: one form of mutation may be via genome fractionation, as duplicate meiosis gene copies return to single copy (Langham et al. 2004; Lloyd et al. 2014; Gonzalo et al. 2019). This subject has been extensively reviewed by (Katche and Mason 2023).

1.5 Genetic control of meiosis in neopolyploids

Meiosis must be tightly regulated for polyploid evolution and survival of progeny to subsequent generations. However, meiotic stabilization has not been extensively studied in polyploid crop species, as only a limited number of studies are available mostly in *B. napus* and wheat (*Triticum aestivum*). Wheat exhibits a diploid-like meiosis, with synapsis and crossover taking place only between homologous chromosomes, despite the presence of ancestrally related and identical homoeologues in the other two subgenomes (Rey et al. 2017). A genetic locus which is responsible for this diploid-like behaviour was identified and mapped on chromosome 5B (Riley and Chapman 1958; Sears 1976). The *Pairing homoeologous* (*Ph1*) locus, which suppresses homoeologous crossovers, was initially identified as a deletion mutant (Sears 1976) of size 59.3 Mb on chromosome 5B (Martín et al. 2018). The *Ph1* locus encodes a duplicated and diverged copy of the meiotic gene *ZIP4* characterized as *TaZIP4-B2* which functions in the suppression of non-homologous crossovers, and which promotes homologous pairing synapsis (Griffiths et al. 2006; Rey et al. 2017, 2018; Martín et al. 2021).

Rapeseed is another crop species in which genetic pairing control loci have been extensively studied. Jenczewski et al. (2003) produced a segregating allohaploid mapping population hybridized from a cross between a high and a low recombination *B. napus* variety, and identified several quantitative trait loci regulating homoeologous pairing. These were identified by measuring and comparing the distribution of chromosome pairing frequencies between the A and C genome of the two contrasting *B. napus* varieties (Jenczewski et al. 2003). One major QTL mapped on chromosome C09 narrowed to a 10-20 cM region was characterized as *Pairing Regulator* in *B. napus* (*PrBn)* which is responsible for suppressing homoeologous pairing and crossovers in *B. napus*

allohaploids (Jenczewski et al. 2003; Liu et al. 2006; Nicolas et al. 2009; Cifuentes et al. 2010). Subsequent meiotic analysis of allotetraploid *B. napus* carrying different versions of the *PrBn* locus showed no influence of *PrBn i*n regulating homoeologous pairing (Grandont et al. 2014), suggesting this locus may only be operating at the allohaploid ploidy level. Higgins et al. (2021) also analysed a segregating mapping population, and identified three QTL which contribute to the regulation of homoeologous recombination in allotetraploid *B. napus*. One major QTL identified as *BnaPh1* on chromosome A09 with 32 – 58% effect on non-homologous recombination event frequencies had five underlying candidate genes including meiosis related *RPA1C* (Replication Protein A 1C) and *MUS81* (MMS and UV sensitive 81) (Higgins et al 2021). However, none of these candidate genes so far identified have been functionally validated. Meiosis in *B. napus*, like all allopolyploid species, is complex, and several genes or interacting genes may be functioning together to regulate homoeologous pairing to achieve stable meiosis.

1.6 Genetic and methylation changes in neopolyploids

During whole genome duplication (WGD) and hybridization in allopolyploids, two ancestrally related subgenomes merge to form a hybrid, a genomic shock usually takes place in form of genomic and epigenetic changes. Song et al. 1(995) was among the first to report the evidence of major genetic changes in resynthesized *B. napus*. Several other studies on neopolyploids and newly synthesized allopolyploids suggest that genomic changes occur rapidly following allopolyploidization in some plant species (Parkin et al. 1995; Udall et al. 2005; Lukens et al. 2006; Gaeta et al. 2007; Szadkowski et al. 2010). Genomic changes which have been reported includes changes in epigenetics, gene expression changes (Jiang et al. 2013; Wu et al. 2018; Pan et al. 2019; Li et al. 2021), chromosome (Udall et al. 2005; Nicolas et al. 2007, 2012; Xiong and Pires 2011), transposon activation (Kashkush et al. 2002; Madlung et al. 2005; Sarilar et al. 2013; Fu et al. 2016), and transcriptomic changes (Marmagne et al. 2010; Xu et al. 2012; Orantes-Bonilla et al. 2023), which are essential to create new gene complexes and rapid evolution (Soltis and Soltis 1999).

Many studies show that novel changes occur in the newly resynthesized progeny as early as the S_0 generation, and accumulate further to subsequent generation. Szadkowski et al. (2010) analyzed three different resynthesized *B. napus* lines, and showed that genetic restructuring is transmitted to further generations, influenced by the cytoplasmic

background inherited from the diploid progenitor. Other studies in allopolyploid *Arabidopsis suecica* have shown evidence of novel gene expression changes occurring in the resynthesized lines, and silencing of some genes in the early generation progeny which were expressed in the parents (Wang et al. 2004). Hu et al. (2023) observed lower gene expression levels in resynthesized *B. napus* compared to their diploid parents. Recently, Li et al. (2022) analyzed gene expression in *B. napus* using long read RNA sequencing, and found that some genes were more highly expressed in natural *B. napus* cultivars compared to the resynthesized lines. Gene expression studies in allopolyploid cotton (*Gossypium* spp.) revealed that natural cotton shows higher transgressive and novel gene expression levels than the diploids and newly formed synthetics (Yoo et al. 2013).
In *Arabidopsis* allotetraploids, changes in gene expression were reported to be primarily as a result of interspecific hybridization rather than genome doubling (Chen 2007). Epigenetic changes which contributes to genetic diversity and diploidization of allopolyploids have played key roles in genome stabilization and evolution of polyploids (Liu and Wendel 2003).

Epigenetic changes, which alter chromatin structure and affect gene expression, have also been observed in newly resynthesized allopolyploids including *B. napus*. Lukens et al. (2006) analyzed early generation resynthesized lines, and detected DNA methylation changes. Hu et al. (2023) observed higher methylation levels in resynthesized *B. napus* compared to its diploid parents. Similarly, significantly higher methylation levels were observed in synthetics compared to natural *B. napus*. Ran et al. (2016) detected higher DNA methylation levels in early generation resynthesized *B. napus* (S_0-S_3). Xiao et al. (2023) observed that transposable elements (TE) methylation levels were negatively correlated with gene expression, and changes in TE methylation levels regulated the expression of some nearby genes. DNA methylation patterns have been shown to contribute to DNA repair and fertility in *B. napus* (Ran et al. 2016; Wang et al. 2016; Yin et al. 2021).

1.7 Aims and scope

Brassica napus have been resynthesized for a long time via interspecific hybridization by crossing the diploid progenitors *B. rapa* and *B. oleracea*, and this protocol has been improved over time by researchers. The production of resynthesized *B. napus* was aimed at increasing genetic diversity of the current rapeseed gene pool, which has been eroded as a result of intensive breeding efforts targeted towards improving its oil quality. Therefore, resynthesis of rapeseed produced from genetically diverse turnips and vegetable-type *B. oleracea* species would be valuable to rapeseed breeders. Although these hybrids could be easily produced, one major drawback is their meiotic instability relative to established *B. napus*. Previous studies have shown that resynthesized *B. napus* has unstable meiosis, unlike established *B. napus*, which affects the fertility and viability of resynthesized *B. napus* across subsequent generations due to poor control of chromosome pairing behaviour (Szadkowski et al. 2010; Xiong et al. 2011).

The question of how established *B. napus* attained meiotic stability is still being investigated by researchers, and many routes to meiotic stabilization in allopolyploids have been proposed. One major hypothesis is through the inheritance of allelic variants present within the diploid progenitors (Cifuentes et al. 2010; Szadkowski et al. 2010; Mason and Wendel 2020; Gonzalo 2022). However, no previous experimental study in *B. napus* has tested this hypothesis using a large collection of resynthesized rapeseed genotypes.

In this thesis, we characterized previously generated diverse sets of resynthesized *B. napus* experimental materials, and assessed fertility, as well as genome stability in these lines. We also analyzed fertility and inheritance of allelic variation in early generation resynthesized *B. napus* lines from diploid *B. rapa* and *B. oleracea* progenitors by identifying homologs of characterised meiosis genes.

The first section of this thesis (Chapter 2) is a review on the relevance of resynthesized *B. napus* as a useful genomic resource for breeding and genomics. Here, we discuss how resynthesized *B. napus* lines have been produced via interspecific hybridization for decades as well as the fertility and meiotic instability of the resulting progenies. We then discuss the agronomic potential of this germplasm in breeding. We shed light on the putative role of meiosis genes, gene expression, and gene regulatory networks in the stabilization of meiosis in polyploid plants. We concluded by discussing the direct use

of resynthesized and semi-resynthesized *B. napus* lines for hybrid breeding and other agronomic traits which have yet to be exploited for the improvement of rapeseed cultivars.

The first study (Chapter 3) investigates the fertility, homozygosity (purity), and genome stability of early and later generations of resynthesized *B. napus* lines. We characterized a total of 140 resynthesized *B. napus* lines comprising 121 domesticated lines produced by crosses between diverse *B. rapa* and vegetable-types *B. oleracea*, as well as 19 wild C-genome species resynthesized lines hybridized between *B. rapa* and wild C genome species (*B. incana*, *B. hilarionis*, *B. montana*, *B. bourgeaui*, *B. villosa*, and *B. cretica*). Then we analyzed these lines for purity (homozygosity), fertility, and genome stability (as measured by the number of CNVs).

The second study (Chapter 4) describes the influence of allelic variation inherited from diploid progenitors on the fertility and genome stability of resynthesized *B. napus* lines. Here, we analyzed 41 early generation (S_1) resynthesized *B. napus* lines hybridized from eight *B. rapa* and eight *B. oleracea* homozygous or inbred parental accessions for copy number variation resulting from homoeologous recombination events, and fertility. We then resequenced eight *B. rapa* and five *B. oleracea* parent genotypes, and assessed 19 resynthesized lines for allelic variation in a list of meiosis gene homologs.

In summary, this thesis aimed to achieve the following goals:

I. Characterization of a large collection of early and late generation resynthesized *B. napus* for homozygosity (purity), and assessing fertility (self-pollinated seeds, seed per ten pods, and pollen viability), as well as genome stability (as measured by the number of copy number variation) in these lines.

II. Testing of the hypothesis that inherited meiotic genes alleles present in the diploid progenitor produced meiotically stable *B. napus*, by using early generation resynthesized (S1) *B. napus* lines.

2.0 Resynthesized Rapeseed (*Brassica napus*): Breeding and Genomics

Elizabeth Ihien Katche and Annaliese S. Mason

2.1 Publication Outline

This review paper discusses and summarizes resynthesized *Brassica napus*, its production via interspecific crosses over the years, and the fertility of hybrids derived as well as useful agronomical traits of this germplasm. It also summarizes meiotic stability challenges of these hybrids, and the putative role of meiosis genes, gene expression, and other gene regulatory networks implicated in stabilizing meiosis. In conclusion, the direct use of resynthesized and semi-resynthesized *Brassica napus* for hybrid breeding, and for the improvement of elite rapeseed cultivar in order to introgress other unexplored agronomic traits of interest was discussed.

2.2 Publication

Authors contribution

Elizabeth Ihien Katche wrote the original draft of the review article, Annaliese S. Mason conceptualized and acquired funding as well as reviewed and edited the article

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Resynthesized Rapeseed (*Brassica napus*): Breeding and Genomics

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ABSTRACT

Resynthesized rapeseed lines (2*n* ¼ 4*x* ¼ 38, AACC), which recreate the historical hybridiza- tion between progenitor species *Brassica rapa* (2*n* ¼ 2*x* ¼ 20, AA) and *B. oleracea* (2*n* ¼ 2*x* ¼ 18, CC) to produce *Brassica napus* (2*n* ¼ 4*x* ¼ 38, AACC), have been an important research subject for many years. These lines not only comprise useful genetic resources in rapeseed breeding for the introgression of genetic diversity and many agronomically important traits, but have also increased our understanding of how meiosis evolved in poly- ploid plants. In this review, we discuss and summarize how these lines have been produced via interspecific crosses over several decades and the resulting fertility and agronomically useful traits in this germplasm, as well as meiotic instability issues in these hybrids, and the putative role of meiosis genes, gene expression and other gene regulatory networks in the stabilization of meiosis. Finally, we discuss the direct use of resynthesized and semi-resyn- thesized *Brassica napus* for hybrid breeding as well as for the study of other unexplored agronomic traits of interest for the improvement of elite rapeseed cultivars.

KEYWORDS

Fertility; genome stability; Interspecific hybridization; meiosis genes; polyploidy; resynthesized *B. napus*

I. Introduction

Polyploidy is the heritable condition of possessing more than two sets of chromosomes, and is a major feature of plant genome evolution (Alix *et al.*, 2017). Polyploidy is ubiquitous across both plant and animal kingdoms, with a few exceptions (Van De Peer *et al.*, 2017). In particular, all angiosperms have experienced one or more rounds of whole genome duplication (WGD) in their evolutionary history (Jiao *et al.*, 2011; Pel,e *et al.*, 2018), and the speciation success of many plant species has been attributed to polyploidy and hybridization (Rieseberg, 1997; Ainouche *et al.*, 2004; Soltis *et al.*, 2014; Pel,e *et al.*, 2018). Polyploidy is clas- sified into allopolyploidy, where two (or more) genomes from different species come together in the new polyploid, and autopolyploidy, in which more than one approximately equivalent homologous copy of each chromosome is present within a species (Bomblies, 2023). These categories are not absolute, and gradients of relatedness exist between polyploid subgenomes from 100% identity (from chromosome doubling within a single individual) to relatively extreme divergence, as well as more complex inter- mediate states which can arise in the generations fol- lowing the initial polyploidy event (for review see Mason and Wendel, 2020).

Polyploidy has been widely studied since the 1950s, with a primary focus on evolutionary and phylogen- etic analyses of existing polyploid plant species. However, there are still many unanswered questions about polyploidy, hybridization and speciation proc- esses. Mechanistic questions such as how newly formed polyploids stabilize meiosis (Pel,e *et al.,* 2018), when and why genomic rearrangement occurs in some polyploids and not in others (Soltis *et al.*, 2014), and how gene expression regulation changes following polyploidy (Yoo *et al.*, 2013) are difficult to address from a phylogenetic perspective. However, recreating speciation events in shallow evolutionary time has great potential to provide insights into some of these processes. One way to do this is to make interspecific hybrids by crossing two divergent extant parents. Interspecific hybrids have a long history of being used for the study of genome evolution and polyploidy (Hegarty *et al.*, 2006; Zhao *et al.*, 2018; Palacios *et al.*, 2019), meiotic stability (Szadkowski *et al.*, 2010; Ferreira de Carvalho *et al.*, 2021; Xiong *et al.*, 2021) as well as the introgression of useful traits into elite lines (Akbar, 1990a; Diederichsen and Sacristan, 1996; Eickermann and Ulber, 2011).

Resynthesized lines are potentially of great interest in breeding. These may show particular promise for

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hybrid breeding because strong breeding selection for agronomically useful traits by breeders has eroded the gene pools of many cultivars (Girke *et al.*, 2012b; Jesske *et al.*, 2013; Szała *et al.*, 2016). Hence, a new, genetically diverse germplasm pool produced from resynthesized lines can be utilized to produce highly heterotic hybrids with preexisting elite lines. Another useful breeding strategy is to exploit the diploid pro- genitors of crop species and/or their wild crop rela- tives as sources of novel favorable alleles for the improvement of current breeding lines (Udall *et al.*, 2004) as well as for the broadening of genetic diversity (Girke *et al.*, 2012b; Rahman *et al.*, 2015; Wu *et al.*, 2015). Resynthesized lines have been studied for the introgression of several agronomically useful traits such as disease resistance (Niemann *et al.*, 2017; Mahmood *et al.*, 2020; Kawasaki *et al.*, 2021) yield/he- terosis (Udall *et al.*, 2004; Girke *et al.*, 2012a; Jafarzadeh *et al.*, 2016), drought resistance (Jiang *et al.*, 2019), grain size (Ma *et al.*, 2016; Yan *et al.*, 2018), and early flowering (Schranz and Osborn, 2000; Rahman *et al.*, 2011).

Resynthesized lines have been produced in a num- ber of plant species, most commonly in wheat (Gorafi *et al.*, 2018; Mirzaghaderi *et al.*, 2020), *Arabidopsis* (Comai *et al.*, 2000; Madlung *et al.*, 2002; 2005; Beaulieu *et al.*, 2009), and *Brassica* species (Abel *et al.*, 2005; Mason *et al.*, 2012; Jesske *et al.*, 2013; Gaebelein *et al.*, 2019; Katche *et al.*, 2021). Resynthesized or syn- thetic wheat has been widely produced and investi- gated over the last 50 years (Maan and McCracken, 1968; Kempanna and Seetharam, 1972; Duca *et al.,* 1980; Fu *et al.*, 2022), and despite the challenges involved due to its large genome size and complex genome structure (Guan *et al.*, 2020; Walkowiak *et al.*, 2020) wheat is still one of the best-studied allopoly- ploid systems (Jenczewski and Alix, 2004). In recent years, allopolyploid and autopolyploid *Arabidopsis* rel- atives have also attracted substantial research attention as models for synthetic polyploid formation: these offer substantial advantages of small genome size, rapid life cycles and impressive quantities of available genetic and genomic information (for review see Lloyd and Bomblies, 2016). *Brassica napus* (2*n* ¼ 4*x* ¼ 38, AACC) is however interesting by itself as a very young species, arising in the last 10,000 years via human agricultural selection (Chalhoub *et al.*, 2014) and comprising the closest crop relative to *Arabidopsis* (Mason and Snowdon, 2016; Hu *et al.*, 2021). It can also be readily synthesized from highly diverse extant progenitor species *B. rapa* (2*n* ¼ 2*x* ¼ 20, AA) and *B. oleracea* (2*n* ¼ 2*x* ¼ 18, CC) (Harberd and McArthur 1980; FitzJohn *et al.*, 2007), which also contain many cultivated crop types, offer- ing a dual perspective on both allopolyploid formation and crop domestication and selection processes (Mason and Snowdon, 2016).

Several studies have investigated genomic and proteomic changes, DNA methylation, and meiosis in resynthesized *B. napus* in order to understand the evolutionary changes that occur during the formation of polyploid species Song *et al.*, 1995; Lukens *et al.*, 2006; Gaeta *et al.*, 2007; Szadkowski *et al.*, 2010; Kong *et al.*, 2011; Xiong *et al.*, 2011b). Several authors have hypothesized that the success and diversification of polyploid lineages could be attributed to the occur- rence of rapid genome changes which, might have led to the acceleration of evolutionary processes in newly synthesized polyploids, especially in *Brassica* (Song *et al.*, 1995; Schranz and Osborn, 2000; Soltis *et al.*, 2015). Investigating questions related to polyploidy, hybridization, genome evolution and meiotic stabiliza- tion in *Brassica* polyploids has become easier with improved methods for interspecific hybrid production, ploidy manipulation and the availability of a full suite of molecular and cytogenetic tools as well as reference genome information and resequencing data (Mason and Snowdon, 2016). Recently, genetic manipulation techniques such as genome editing have also been proposed to facilitate the neo-domestication of new *Brassica* polyploids and wild species by editing genes related to domestication, genome stability, and recom- bination (Hu *et al.*, 2021).

Genome stability has been a major challenge in newly synthesized *B. napus* lines. Resynthesized *Brassica napus* has been shown to be meiotically unstable, unlike natural *B. napus* (Jenczewski *et al.*, 2003; Szadkowski *et al.*, 2010; Xiong *et al.*, 2011b; Nicolas *et al.*, 2012; Grandont *et al.*, 2014; Rousseau- Gueutin *et al.*, 2017). However, only a few studies have attempted to understand the genetic factors that may be responsible for this phenomenon (Ferreira de Carvalho *et al.*, 2021; Katche *et al.* 2021, preprint; Xiong *et al.*, 2021), which may be of special interest for the study of the control of meiosis in newly formed polyploids (Pel,e *et al.*, 2018; Higgins *et al.*, 2021).

In this review, we aim to comprehensively sum- marize what studies in resynthesis of *B. napus* have taught us about how two genomes come together to form a new, stable allopolyploid species. We also aim to summarize studies which have produced resynthe- sized *B. napus* lines via interspecific hybridization, and how this germplasm has been utilized for the

study and introgression of agronomically useful traits into cultivated rapeseed.

II. Resynthesized *Brassica napus*: history and methods of resynthesis

Resynthesis is the process of reproducing an already existing species from its progenitor species (Katche *et al.*, 2019). Resynthesized *B. napus* can be produced by interspecific hybridization between *B. rapa* and *B. oleracea* via sexual crosses between diploids or tetraploid parents (Inomata, 1977; Olsson, 1960), and by protoplast fusion of diploid progenitors (Sundberg and Glimelius, 1986; Terada *et al.*, 1987; Jourdan *et al.*, 1989). Resynthesis of *B. napus* is useful to broaden the genetic diversity of established *B. napus*, which has limited genetic diversity as a new allopoly- ploid formed from only one or two hybridization events between diploid progenitors (Chalhoub *et al.*, 2014), and whose genetic variation has been further eroded as a result of breeding for high quality rape- seed oil with low glucosinolate and low erucic acid content (Girke *et al.*, 2012a; Rahman *et al.*, 2015). The production of resynthesized *B. napus* is hence import- ant to introduce desirable agronomical traits from diploid progenitors into rapeseed.

Possibly the earliest report of resynthesis of *B. napus* is U (1935), from which paper we also have the "Triangle of U" establishing the genomic relation- ships between the three diploid (*B. rapa, B. nigra* and *B. oleracea*) and three allotetraploid *Brassica* species (*B. juncea*, *B. napus* and *B. carinata*). U (1935) crossed nine different varieties each of *B. rapa (B. campestris*) and *B. oleracea* and produced four F_1 hybrid plants: two putative triploids ($2n = \sim 28$), one haploid ($2n = 19$) and one spontaneous allotetraploid *B. napus* plant ($2n = 38$). Afterwards, several other attempts were made to produce resynthesized *B. napus* using different methods that included applica- tion of chemicals into the ovary or peduncle (Honma and Heeckt, 1960; Namai, 1971), repeated or mixed pollinations (Sarashimi, 1967), style excision or graft- ing (Hosoda *et al.* 1963; Namai, 1971) and *in vitro* fertilization (Kameya and Hinata 1970; Inomata 1977, 1983; reviewed by Chen and Heneen, 1990), that resulted in both failed and successful attempts. Many of these methods were adopted in order to bypass the reproductive incompatibility barriers that are common in sexual hybridization. However, some of these meth- ods also have their limitations. For example, although chemical application to induce male sterility shortens the time required to obtain viable interspecific hybrids, it is difficult to induce male sterility without an adverse effect on female fertility (McRae, 1985), and many of these attempts have failed. Grafting as a means of hybridization on the other hand is signifi- cantly more efficient in fruit trees and flowering shrubs with longer lifecycles, as these take a long time to grow using conventional plant breeding methods (Zhou and Liu, 2015). Resynthesized *B. napus* has also been synthesized from crosses between *B. rapa* and *B. oleracea* by sexual hybridization followed by embryo culture (Sarashima, 1967; Takeshita *et al.*, 1980), ovary culture (Inomata 1977, 1978, 1983; Takeshita *et al.*, 1980); (Inomata 1977, 1978, 1983, 1985; Takeshita *et al.* 1980), or ovary-embryo culture (Matsuzawa, 1978).

The two major methods by which resynthesized *B. napus* can be produced are via somatic fusion and sexual hybridization, both of which methods have dis- tinct advantages and disadvantages. Protoplast fusion offers the advantage of novel cytoplasmic combina- tions and retention of heterozygosity in the resulting progeny, although these potentials have not been fully utilized (Ozminkowski and Jourdan, 1994). In add- ition, somatic fusion can overcome incompatibility barriers and ensures the transfer of useful traits between sexually incompatible species (reviewed by Katche *et al.*, 2019). On the other hand, sexual hybridization to resynthesize *B. napus* involves inter- specific hybridization by sexual crosses between dip- loid or induced tetraploid parents, which is technically easier and does not require sterile laboratory condi- tions (Olsson, 1960; Inomata, 1978; Akbar 1990a).

Somatic fusion in resynthesized *B. napus* involves the fusion of protoplasts obtained either from leaf or hypocotyl tissue from both diploid parents (Sundberg and Glimelius, 1986; Jourdan *et al.*, 1989). Between the late 1980s and 1990s, many studies produced resynthesized *Brassica napus* using leaf protoplasts from young plants of both *B. oleracea* and *B. rapa* (Ozminkowski and Jourdan, 1993; Ozminkowski and Jourdan, 1994). Sundberg and Glimelius (1986) pro- duced resynthesized *B. napus* from two different culti- vars of each of *B. oleracea* and *B. rapa* (*B. campestris*) using leaf protoplasts from one parent and hypocotyl protoplasts from the other, and obtained four hybrid plants from 450 calli. Protoplast fusion has been used in *Brassica* is to manipulate traits such as cytoplasmic male sterility (CMS), which is controlled by mito- chondria, and photosynthetic herbicide resistance, which is controlled by chloroplasts (Yarrow *et al.*, 1986; Menczel *et al.*, 1987; Barsby *et al.*, 1987b, 1987a). Jourdan *et al.* (1989) produced triazine-

resistant *B. napus* plants by somatic hybridization using leaf protoplasts from a male-sterile *B. oleracea* ssp. *botrytis* line carrying the Ogura radish-derived male sterile cytoplasm (Pellan-Delourme and Renard, 1988; Delourme *et al.*, 1994), and a hypocotyl from an atrazine-resistant *B. rapa* (*B. campestris*) in two different experiments, and obtained 50 plants from 28 calli. By contrast, (Robertson *et al.*, 1987) using a similar technique produced only one atrazine-resistant resynthesized *B. napus* line from the leaf protoplast of a male sterile *B. oleracea* var. *italica* and a hypocotyl protoplast from an atrazine resistant *B. rapa* (*B. cam- pestris*). Hypothetically, differences in the number of resynthesized *B. napus* lines produced using protoplast fusion might be as a result of genotypic differences as well as experience or protocols used for the tissue culture experiments.

Resynthesized *B. napus* is most commonly produced by crossing (hand-pollination) between *B. rapa* and *B. oleracea* progenitor species to produce the haploid F_1 hybrid, followed by subsequent induction of chromosome doubling using colchicine to produce R_0 resynthesized *B. napus* (Abel *et al.*, 2005; Girke *et al.*, 2012a; Jesske *et al.*, 2013a). U (1935) first made reciprocal crosses between *B. rapa* and *B. oleracea* but only produced F_1 hybrids with *B. rapa* as the female par- ent. Olsson (1960) made reciprocal crosses between both diploid and tetraploid *B. campestris* (*B. rapa* ssp. *oleifera* (turnip rape)) and *B. oleracea* by hand pollination, and obtained eight times more F_1 plants with tetraploid × tetraploid crosses (133 F_1 plants from 22,884 bud pollinations) than with diploid × diploid crosses (16 F_1 plants from 10,395 bud pollinations). The reciprocal tetraploid cross *B. oleracea* × *B. rapa* ssp. *oleifera* produced 130 F_1 plants, compared to no plants with diploid crosses. However, both diploid and tetraploid parental crosses involving *B. rapa* ssp. *rapa* (turnip) produced significantly lower seed set compared to turnip rape (Olsson, 1960). Frandsen (1947) crossed tetraploid *B. rapa* (*B. campestris*) and *B. oleracea* and obtained 65 hybrid F_1 plants from 3,000 bud pollinations while Hoffman and Peters (Hoffmann and Peters, 1958) obtained no plants from more than 9,000 bud pollinations. These differences in the number of F_1 plants obtained by these authors is explained in part by the differences in parental genotypes used in the crosses (Olsson 1960). In the twentieth century, most hybrids produced from *B. oleracea* × *B. rapa* crosses were largely sterile (U, 1935; Nishi *et al.,* 1959; Olsson, 1960; Hosoda *et al.,* 1963), with only a few exceptions (Hoffmann and Peters 1958; Takeshita *et al.*, 1980). Takeshita *et al.*, (1980) for example obtained a higher rate of F_1 hybrids in *B. oleracea* × *B. rapa* than in the reciprocal direction using ovule culture. So far, only heterozygous geno- types were used for the production of interspecific crosses. Years later, Abel *et al.*, (2005) produced 197 homozygous resynthesized lines from crosses between homozygous cultivars and varieties (21 *B. rapa* and 16 *B. oleracea*), and obtained 3,485 vital embryos from 9,514 pollinated buds using *B. rapa* as the maternal parent.

Most studies in the present century have focused on producing resynthesized *B. napus* for the improvement of current *B. napus* elite cultivars, specifically in order to introduce new genetic diversity (Lu *et al.*, 2001; Seyis *et al.*, 2003; Girke *et al.*, 2012a; Jesske *et al.*, 2013; Sosnowska and Cegielska-Taras, 2014; Hilgert-Delgado *et al.*, 2015; Rahman *et al.*, 2015; Szała *et al.*, 2016) or to introgress agronomically important traits (Diederichsen and Sacristan, 1996; Rahman, 2001; Child *et al.*, 2003; Zhang and Zhou, 2006; Zhao *et al.*, 2009; Schaefer-Koesterke *et al.*, 2017; Ding *et al.*, 2019; Jiang *et al.*, 2019). Several studies have also addressed evolutionary questions related to how meiosis evolved in established *B. napus* (Song *et al.*, 1995; Jenczewski *et al.*, 2003; Nicolas *et al.*, 2008; Szadkowski *et al.*, 2010; Ferreira de Carvalho *et al.*, 2021; Higgins *et al.*, 2021). Many of these studies used sexual hybridization between *B. rapa* and *B. oleracea* to produce F_1 hybrids which were then colchicine-treated to induce chromo- some doubling before self-pollination to produce subse- quent generations.

Studies comparing the fertility of resynthesized *B. napus* with natural *B. napus* mostly show that resynthesized *B. napus* lines are lower in fertility (Xiong *et al.*, 2011b; Guo *et al.*, 2016; Rousseau-Gueutin *et al.*, 2017; Ferreira de Carvalho *et al.*, 2021), and that fertility decreases further in subsequent generations following self-pollination. Only a few studies have observed comparable or higher fertility to established *B. napus*, usually in a few resynthesized individuals (Girke *et al.*, 2012a; Karim *et al.*, 2014). From all of these studies, it is clear that the fertility of resynthesized lines is genotype-dependent, and that early generation resynthesized lines may not all survive to subsequent self-pollinated generations due to frequent sterility.

III. Agronomic potential of resynthesized *Brassica napus*

One known example of resynthesized *B. napus* with economic use which has become an agriculturally marketable and economically useful cultivar is the

Japanese 'Hanakkori' produced by a cross between the Chinese vegetable *B. rapa* var. *utilis* (AA, 2*n* ¼ 20) and *B. oleracea* var. *italica* (CC, 2*n* ¼ 18) (Fujii and Ohmido, 2011). Hanakkori is famous for its easy culti- vation and high nutritional value (Fujii and Ohmido, 2011).

In research, many studies on resynthesized *B. napus* have investigated its potential in breeding for agronomically important traits, particularly as established *B. napus* cultivars are limited in genetic and phenotypic diversity (Seyis *et al.*, 2003; Hasan *et al.*, 2006; Guo *et al.*, 2016). The diploid progenitor species *B. rapa* and *B. oleracea* as well as wild relative C-gen- ome species such as *B. incana*, *B. hilarionis*, *B. cretica*, *B. insularis* are highly genetically and morphologically diverse (Cheng *et al.*, 2016b), as well as abundant in agronomically useful traits which could possibly be introgressed into established *B. napus* via interspecific hybridization. Other reviews have covered many of these agronomic traits in other *Brassica* species and hybrids (Warwick *et al.*, 2009; Katche *et al.*, 2019; Quezada-Martinez *et al.*, 2021) although not specifically in resynthesized *B. napus*.

A. Disease resistance

Diseases caused by pathogens are a major challenge in rapeseed production (Zheng *et al.*, 2020). Chemicals and other agricultural control measures are expensive, inconsistent and less environmentally friendly (Diederichsen and Sacristan, 1996; Neik *et al.*, 2017; Ding *et al.*, 2019), such that resistance breeding via effective host resistance is the most cost effective and reliable means of disease control (Neik *et al.*, 2017). Resistant forms of *B. napus* to many major diseases are either lacking or limited and most often race-spe- cific (Diederichsen and Sacristan, 1996; Rygulla *et al.*, 2007a; Ding *et al.*, 2019), but *B. rapa* and *B. oleracea* are good sources of resistance to many common pathogens that affect rapeseed production (for review see Warwick *et al.*, 2009; Katche *et al.*, 2019; Quezada-Martinez *et al.*, 2021). Several studies have produced resynthesized *B. napus* lines resistant against diseases common in rapeseed such as clubroot (Diederichsen and Sacristan, 1996; Rahman *et al.*, 2014; Niemann *et al.*, 2017; Kawasaki *et al.*, 2021), Verticillium wilt (Happstadius *et al.*, 2003; Eynck *et al.*, 2007; Rygulla *et al.*, 2007b, 2007a, 2008; Obermeier *et al.*, 2013), Sclerotinia stem rot (Ding *et al.*, 2013; Mei *et al.*, 2013; 2015; Ding *et al.*, 2019) and blackleg (Crouch *et al.*, 1994; Leflon *et al.*, 2007; Yu *et al.*, 2012, 2013).

1. Clubroot disease

Clubroot disease, named for the symptom of swollen or club-shaped roots, is caused by the soilborne obli- gate biotrophic protist *Plasmodiophora brassicae* Woronin belonging to the class *Phytomyxea* (*Plasmodiophorids*) of the Eukaryotic Kingdom Rhizaria (Hwang *et al.*, 2012). Clubroot disease is one of the most damaging diseases of *B. napus* worldwide, and is of special concern in Europe and North America (L€ ders *et al.*, 2011). Spores of *P. brassicae* can survive in the soil for a long period, thereby mak- ing it difficult to control the disease by cultural practi- ces or chemical treatment (Voorrips, 1995). Cultural practices are traditional agricultural management tech- niques and methods used by farmers or breeders to optimize crop yield and productivity. Cultural practi- ces for the control of plant pest and diseases include use of disease-free seeds, time of planting, depth of seeding, crop rotation, tillage, adequate and balanced plant nutrition, and flooding (Kharbanda and Tewari, 1996). Breeding clubroot-resistant *B. napus* cultivars (combined with good cultural practices) is the most effective means for long-term management of this dis- ease (Rahman *et al.*, 2014). Clubroot-resistant lines have been found in turnips (*B. rapa* ssp. *rapa*) and in *B. oleracea* and its wild accessions, as well as in a few other *Brassica* species (Crute *et al.*, 1983; Toxopeus *et al.*, 1986; Crisp *et al.*, 1989; Hasan *et al.*, 2012). Diederichsen and Sacristan (1996) generated interspecific hybrids between one clubroot-resistant *B. rapa*, and a resistant and a susceptible *B. oleracea* variety: resynthesized lines with two resistant parents showed a very broad and effective resistance against isolates that were virulent on all *B. napus* cultivars. Kawasaki *et al.*, (2021) produced clubroot-resistant resynthesized *B. napus* by interspecific crosses between *B. oleracea* and *B. rapa* (Chinese cabbage) as donors of two club-root resistant loci *crr1* and *crr2*, and selected for these two *crr* genes using marker assisted selection in subsequent backcross generations to produce clubroot-resistant, semi-resynthesized *B. napus* lines (Kawasaki *et al.*, 2021). It has been suggested that clubroot resistance from *B. rapa* might be more effective in controlling clubroot disease than resistance from *B. oleracea* (reviewed by Rahman *et al.*, 2014), but the combination of resistance from both parent species in resynthesized rapeseed also seems clearly advantageous.

2. Sclerotinia stem rot

Sclerotinia stem rot caused by the necrotrophic fungus *Sclerotinia sclerotiorum* affects the stem and pods of rapeseed (Hind *et al.*, 2003) and is a major threat to

seed yield and quality (Bolton *et al.,* 2006; Koch *et al.*, 2007; Sharma *et al.,* 2015). The most sustainable way of controlling *Sclerotinia* stem rot is by breeding resistant rapeseed varieties (Ding *et al.*, 2013), and although resistance is limited in current rapeseed germplasm, cultivated and wild types of progenitor species *B. oleracea* are good sources of resistance against *Sclerotinia* (Mei *et al.*, 2011; Ding *et al.*, 2013, 2019). Ding *et al.*, (2013) screened 55 resynthesized rapeseed lines derived from seven wild and two culti- vated *B. oleracea* types, and found resynthesized lines showed stronger stem resistance compared with the partially resistant *B. napus* control cultivar. Similarly, Mei *et al.*, (2011) screened 68 accessions in six *Brassica* species including 47 accessions of wild and cultivated *B. oleracea* accessions for leaf and stem resistance to *S. sclerotiorum*, and found high levels of resistance in wild *B. oleracea* coenospecies (sharing the C genome), especially *B. rupestris*, *B. incana*, *B. insularis* and *B. villosa*. Ding *et al.* (2019) evaluated 37 resynthesized lines produced from crosses with or without sclerotinia resistant *B. rapa* and/or *B. olera- cea*, and observed a 2.7 fold increase in *Sclerotinia* resistance in one resynthesized line compared to a partially resistant rapeseed variety. Mei *et al.*, (2015) also transferred *Sclerotinia* resistance into rapeseed via hexaploids (AACCCC) derived from crosses between *B. incana* (a wild relative of *B. oleracea*) and a Chinese rapeseed cultivar, followed by subsequent backcrossing and marker assisted selection to select for *Sclerotinia*-resistant individuals. Out of 100 back-crossed F_2 individuals, one individual with 38 chro-mosomes and almost 2-fold higher resistance compared to the *B. napus* check cultivar was identified.

3. Verticillium wilt

Verticillium wilt disease is caused by the fungus *V. longisporum*, a soilborne pathogen which infects through the roots by direct penetration of the epider-mal cells or through open wounds (Happstadius *et al.*, 2003). This disease threatens rapeseed production especially in Northern Europe (Karapapa *et al.*, 1997; Happstadius *et al.*, 2003). Rapeseed cultivars with improved resistance offer one way to minimize losses caused by the pathogen. However, no known resistant cultivar exists within the rapeseed germplasm (Happstadius *et al.*, 2003). Interestingly, valuable sour-ces of resistance have been identified in *B. oleracea* and successfully transferred via interspecific hybridiza- tion from the *B. oleracea* progenitor C genome donor into resynthesized *B. napus* (Happstadius *et al.*, 2003; Rygulla *et al.*, 2007a, 2007b). Erucic acid is a long chain monosaturated fatty acid which is detrimental to human health (Wang *et al.*, 2022). In order to pro- duce a *Verticillium*-resistant resynthesized *B. napus* line, Rygulla *et al.*, (2007a) identified resistance against *V. longisporum* in a zero erucic acid *B. olera- cea* convar. *capitata* variety, and generated three resynthesized lines by crossing the resistant genotype with two accessions of *B. rapa ssp. oleifera* with zero erucic acid and one double high (high erucic acid and high glucosinolate content) quality yellow-seeded *B. rapa* ssp. *trilocularis* variety. A high level of resistance was found in one of the resynthesized lines compared with both parental accessions and *B. napus* cultivar controls (Rygulla *et al.*, 2007a). Eynck *et al.* (2009) also screened 1,230 accessions of *B. napus*, 180 *B rapa*, and 33 *B. oleracea* accessions for susceptibility to *V. longisporum*, in order to identify potentially resistant parental genotypes to generate resynthesized *B. napus* lines with improved resistance to *V. longispo-rum*. Enhanced resistance to *V. longisporum* was observed in the same resynthesized lines generated by (Rygulla *et al.*, 2007a;b; Eynck *et al.*, 2009). Obermeier *et al.*, (2013) analyzed 214 homozygous lines in a doubled-haploid mapping population produced from a cross between a partially resistant rapeseed cultivar (Express617) and a *Verticillium*-resistant resynthesized line (R53), and identified a major *Verticillium*-resist-ant QTL contributed by R53 on chromosome C5 as well as markers flanking this QTL in four other doubled-haploid populations derived from crosses between other resynthesized lines and the check *B. napus* cultivar.

4. Blackleg disease (stem canker, Phoma)

Blackleg or Phoma disease caused by the fungal pathogen *Leptosphaeria maculans* is a common disease of *Brassica* crops and is responsible for severe yield losses worldwide in *B. napus* production (Gugel and Petrie, 1992; Fitt *et al.*, 2006). The most effective way to control blackleg disease is by breeding resistant cul-tivars (Rimmer and Van Den Berg, 1992). Resistance to blackleg disease has been identified in many *Brassica* species including *B. rapa* (Mithen *et al.*, 1987; Leflon *et al.*, 2007), *B. napus* L. (Rimmer and Van Den Berg, 1992) and several B-genome (Ch'evre et al., 1996, 1997; Plieske *et al.*, 1998; Christianson *et al.*, 2006) and C-genome species (Mithen and Lewis, 1988) (as reported by Yu *et al.*, 2013). Leflon *et al.* (2007) generated resynthesized lines by interspecific crosses between a resistant *B. rapa* and a susceptible double haploid *B. oleracea* followed by a cross with

resistant *B. napus* cv. *Darmor* cultivar to produce 300 doubled-haploid lines, out of which 31 lines were subsequently selected for fertility and blackleg resistance. Yu *et al.* (2012) produced resynthesized allotriploids (2*n* ¼ AAC) from a cross between a resistant *B. rapa* ssp. *sylvestris* and a *B. napus* cultivar, and successfully transferred this blackleg resistance into *B. napus* via backcrossing. Crouch *et al.* (1994) generated resynthesized *B. napus* lines from crosses between six accessions belonging to four subspecies of *Brassica rapa*, including three accessions of *B. rapa* ssp. *sylvestris* and *B. oleracea* as well as between synthetic *B. napus* F_1 hybrids and three different *B. napus* cultivars. Synthetic lines derived from two wild accessions of *B. rapa*, and their F_1 hybrids with oilseed rape cultivars, both expressed high levels of resistance to *L. maculans* in glasshouse experiments, with one of the lines also expressing high levels of resistance after exposure to different pathogens in the field (Crouch *et al.*, 1994).

B. Yield and heterosis

One of the ways to improve seed yield in oilseed rape is through heterosis (Wolko *et al.*, 2019). Heterosis or hybrid vigor has been described as a phenomenon in which offspring (F_1) or interspecific hybrids exhibit better agronomic characters such as increased bio- mass, higher seed yield, rapid development, increased tolerance to stress conditions, and higher resistance than their parents (Fujimoto *et al.*, 2018). Diversity in the *B. napus* gene pool is a requirement for successful hybrid breeding programs, due to a general positive correlation between heterosis and genetic distance (Jesske *et al.*, 2013). However, *B. napus* has a narrow gene pool as a result of intensive breeding efforts and few hybridization events between its diploid *B. rapa* and *B. oleracea* progenitors (Cowling, 2007; Chalhoub *et al.*, 2014; Mason and Snowdon, 2016). Hence, the diploid progenitor species *B. rapa* and *B. oleracea* rep- resent unexplored sources of genetic diversity which can be introgressed into *B. napus* (Udall *et al.*, 2004), which can also be used to develop synthetic rapeseed pools to exploit heterosis effects in hybrid breeding.

Several studies have shown that the use of resynthesized *B. napus* lines in crosses with winter and/or spring *B. napus* cultivars can result in increased hybrid yield and/or mid-parent heterosis (Udall *et al.*, 2004; Seyis *et al.*, 2006; Zhao *et al.*, 2009; Girke *et al.*, 2012a). Girke *et al.* (2012a) produced interspecific hybrids from crosses between resynthesized lines of diverse genetic backgrounds and male-sterile winter oilseed tester lines, and these interspecific hybrids showed higher mean yield than the *B. napus* check cultivars. This suggests the existence of alleles contributing to increased yield in the parental resynthesized lines (Girke *et al.*, 2012a). Similarly, Zhao *et al.* (2009) produced 64 interspecific *B. napus* hybrids from crosses between 4 resynthesized *B. napus* lines and 4 *B. napus* cultivars, and obtained a higher mid-parent heterosis in the F_1 hybrids compared to *B. napus* cultivars. Udall *et al.*, (2004) produced segregating doubled haploid lines from crosses between an elite adapted *B. napus* line and two types of nonadapted germplasm: one Chinese winter cultivar and a resyn- thesized *B. napus* obtained from a cross between *B. rapa* cv. Reward and a rapid cycling *B. oleracea*. A significantly higher seed yield was observed in some of the test crosses compared to both the initial hybrid combination and to all of the commercial check culti- vars. Jesske *et al.* (2013) generated 44 resynthesized lines from crosses between *B. rapa* and wild *B. olera- cea* ssp. *oleracea* as well as 10 wild *Brassica* species in order to investigate the possibility of expanding gen- etic diversity and improving yield in the current *B. napus* gene pool. (Jesske *et al.*, 2013) observed lower yield in resynthesized lines from wild nonadapted *B. oleracea* parents compared to those produced from domesticated *B. oleracea* parents, although high-yield- ing hybrids were produced when these lines were sub- sequently crossed with adapted *B. napus* cultivars. Seyis *et al.* (2006) produced hybrids from male-sterile double-low spring rapeseed lines crossed with nine high erucic-acid content resynthesized lines derived from crosses between *B. oleracea* cultivars and *B. rapa* spp. *trilocularis* as well as three old spring *B. rapa* cul- tivars. The yield potentials of the resynthesized lines when tested in multiple field locations compared to check cultivar *B. napus* controls were dependent on the interactions between genotype and environment (Seyis *et al.*, 2006).

C. Insect resistance

One of the biggest challenges facing rapeseed produc- tion is the control of insect pests, which can cause substantial yield losses (Zheng *et al.*, 2020). The most economically important pests of rapeseed/canola are flea beetles (*Phyllotreta spp.* and *Psylliodes chrysoce- phala*), weevils (*Ceutorhynchus obstrictus syn. C. assimilis*, *C. napi*, *C. Pallidactylus* and *C. picitarsis*), pollen beetles (*Meligethes aeneus syn. Brassicogethes aeneus*), flies and midges (*Delia radicum* and *Dasineura brassicae*), moths (*Plutella xylostella* and *Mamestra configurata*) and aphids (*Brevicoryne*

brassicae and *Myzus persicae*). Insect pests are commonly controlled by using insecticides, but the increasing occurrence of insecticide-resistant populations and the socio-economic context opposes the sole use of insecticides as an efficient and long-term means of control of insect pests (Herv‚e, 2018). An alternative crop protection strategy is to exploit the natural resistance of rapeseed as a tool for integrated pest management. However, no insect-resistant rapeseed cultivar is currently known (Obermeier *et al.*, 2022). Resynthesized lines of *B. napus* have potential to broaden the genetic variability and may improve resistance to insect pests (Schaefer *et al.*, 2017).

Schaefer *et al.* (2017) evaluated the susceptibility to rape stem weevil *Ceutorhynchus napi* Gyll. (*Coleoptera*, *Curculionidae*) of three cultivars, one breeding line, and five resynthesized lines of oilseed rape in the field under free insect predation choice conditions. One resynthesized line (S30) showed enhanced resistance against *C. napi* compared to *B. napus* cultivars and the other resynthesized lines investigated, as indicated by fewer larvae and slow larval development (Schaefer *et al.*, 2017). In a follow-up study, Schaefer-Koesterke *et al.* (2017) investigated long stem length and glucosinolate content as poten- tial mediators of resistance against *C. napi* in five resynthesized lines and three cultivars of oilseed rape, and found more resistance to *C. napi* in resynthesized line S30 compared to commercial *B. napus* cultivars. (Eickermann and Ulber, 2011) screened nine resynthesized rapeseed lines and six *B. napus* cultivars for resistance against *C. pallidactylus* (cabbage stem weevil) in both laboratory and semi-field conditions using larvae numbers and glucosinolate levels as potential indicators of resistance. Eickermann and Ulber (2011) found fewer eggs of *C. pallidactylus* on five resynthesized lines and swede cultivar "Devon Champion" compared to *B. napus* cultivar "Express," as well as lower numbers of larvae in two resynthesized lines and "Devon Champion" compared to "Express".

Turnip yellows virus (*TuYV*) is aphid-transmitted and causes considerable yield losses in rapeseed and vegetable *Brassica* crops (Greer *et al.*, 2021). Insecticide control of the aphid vector is limited due to insecticide resistance (Bass *et al.*, 2014) and the banning of the most effective pesticides in the EU (The European Commission [EC], 2013). Therefore, plant host resistance is a highly desirable method to control the aphid vector that transmits *TuYV*. The resynthesized rapeseed line "R54" and the Korean spring *B. napus* variety "Yudal" (Hackenberg *et al.*, 2020) are the only known sources of resistance to turnip yellows virus (as reported by Greer *et al.*, 2021). Both resistances were shown to be partial and dominantly inherited, and were mapped to single QTL on chromosome A04 (Dreyer *et al.*, 2001; Juergens *et al.*, 2010; Hackenberg *et al.*, 2020). Greer *et al.* (2021) also produced resistant *TuYV* resynthe- sized *B. napus* lines from reciprocal crosses between *TuYV*-resistant individuals from *B. rapa* and *B. olera- cea* populations. The presence of *TuYV* resistance in the resynthesized lines was confirmed by phenotyping, and resistance QTL from *B. rapa* mapped to chromo- somes A02 and A06 and from *B. oleracea* to chromo- some C05.

D. Pod shatter resistance

Although rapeseed is cultivated as an oil crop globally, it still has some weed-like characteristics such as pods that shatter easily when ripe, which can occur prior to or during harvest (Morgan *et al.*, 1998; Summers *et al.*, 2003). This can cause significant yield losses if harvest is delayed beyond the optimum harvesting season (Price *et al.*, 1996). Yield losses of about 8– 12% of the total seed yield (Kadkol *et al.*, 1984) as well as up to 50% have been estimated by Macleod (1981) in seasons of poor weather conditions prior to and during harvest. Several methods have been used by breeders to reduce the premature shattering of pods including swathing (cutting of the stand to pro- mote premature drying) or the use of desiccant sprays shortly before full pod maturity (Morgan *et al.*, 1998; Summers *et al.*, 2003). However, it is difficult to time precisely these treatments to coincide with the right stage of pod development, which can affect seed qual- ity as well as result in seed contamination via chloro- phyll from immature seeds. Variation in resistance to pod shattering among existing genetic resources or cultivars of oilseed rape is little or nonexistent (Morgan *et al.*, 2000). However, resistance to pod shattering has been found in progenitor *Brassica rapa* and *Brassica oleracea* lines as well as in other mem- bers of the Brassicaceae family such as *Brassica juncea* and *Brassica carinata* (Wang *et al.*, 2007; Raman *et al.*, 2017; Kaur *et al.*, 2020).

Resynthesized *Brassica napus* lines produced by interspecific crosses between *B. oleracea* ssp. *alboglabra* and *Brassica rapa* ssp. *chinensis* have been shown to have a wide range of variation to pod shatter resistance compared to that of rapeseed cultivars (Morgan *et al.*, 1998; Summers *et al.*, 2003). Morgan *et al.*, (1998) developed three resynthesized lines of *Brassica napus* derived from crosses between wild diploid *B.*

oleracea and wild diploid *B. rapa* species, followed by subsequent crosses between the synthetic lines and three rapeseed cultivars to produce three doubled haploid populations of *B. napus*. A wide variation of resistance to pod shattering was found in synthetic lines compared to cultivars of *B. napus* (Morgan *et al.*, 1998). Resistance to pod shattering was proposed to be linked to a failure of cells in the dehiscence zone to degrade as well as to the presence of extra vascular tissue within this zone (Morgan *et al.*, 1998). Later on, Summers *et al.* (2003) developed a *B. napus* population derived from a cross between the doubled haploid breeding line and a synthetic interspecific hybrid of wild *B. oleracea* ssp. *alboglabra* and *B. rapa* ssp. *chinensis*, which was compared to a commercial rapeseed cultivar. Increased shatter resistance in the populations of resynthesized lines compared to the commercial rapeseed cultivar indicated that pod shatter resistance is genetically controlled (Summers *et al.*, 2003). Additionally pruning, plant height and pod character were positively correlated with shatter resistance, while time of sowing (but not any environmental factor) had an influence on shatter susceptibility (Summers *et al.*, 2003). By contrast, Morgan *et al.* (2000) found no correlation between most pod and plant morphological characters. However, this suggests that it would be possible to select for resistance to pod shattering that could be introgressed into *B. napus* cultivars independent of other (possibly negative) agronomic characters from the resynthesized lines (Morgan *et al.*, 2000). A further investigation of the same resynthesized line analyzed by Summers *et al.* (2003) showed that although variation in the pod architecture played little or no role in pod shatter, variation in the dimensions of the dehiscence zone correlated strongly and positively with shatter resistance (Child *et al.*, 2003). Variation in the size of the main vascular bundle was strongly associ- ated to variation in shatter resistance in both resynthe- sized and shatter-susceptible rapeseed cultivars as confirmed by two independent methods (Child *et al.*, 2003). Pod shatter resistance in resynthesized *B. napus* is still understudied, and only very few studies have screened wild *B. rapa* and wild *B. oleracea* for pod shat- ter resistance, while the rest have only analyzed one pod-shatter resistant line. In future, screening a large number of resynthesized lines derived from wild and cultivated diploid *B. rapa* and *B. oleracea* progenitor spe- cies for pod shatter resistance should be considered.

E. Early flowering

Adequate regulation of flowering and flowering time is crucial for crop production, especially for leafy vegetable crops such as *B. rapa* and *B. oleracea* (Schiessl *et al.*, 2017). Knowledge about the impact of flowering time gene variation is therefore crucial for successful vegetable breeding (Schiessl *et al.*, 2017). Early flowering *B. rapa* and/or *B. oleracea* progenitor species have proven to be good sources of early flow- ering and short season duration *B. napus* lines (Karim *et al.*, 2014). For example, *B. rapa* accessions flower and mature earlier than any other species in the *Brassica* U's Triangle (Rahman *et al.*, 2011). The Chinese kale, a variety of *B. oleracea* is one of the ear- liest flowering varieties of *B. oleracea*, although it flowers and matures much later than both *B. rapa* and *B. napus* (Rahman *et al.*, 2011). Early flowering and short duration *B. napus* genotypes have been suc- cessfully resynthesized by interspecific crosses between early flowering varieties of *B. rapa* and *B. oleracea* (Akbar, 1987; 1990a).

Akbar (1990a) produced resynthesized *B. napus* lines through interspecific crosses between three early maturing accessions from both diploid and tetraploid *B. rapa* and *B. oleracea* var. *alboglabra*, and obtained early flowering and maturing resynthesized lines compared to natural *B. napus*. Rahman *et al.* (2011) and Zaman (1989) demonstrated that the C genome of *B. alboglabra* carries early flowering alleles different from those present in the C genome of *B. napus*. Rahman *et al.*, (2011) also produced recombinant inbred lines from interspecific crosses between *B. napus* and *B. oleracea* var. *alboglabra* carrying early flowering alleles, resulting in about a week earlier flowering in the resultant hybrid than in the *B. napus* parent (Rahman *et al.*, 2011). Karim *et al.* (2014) crossed two different *B. rapa* and *B. oleracea* cultivars in five reciprocal cross combinations and produced some early flowering F_3 resynthesized *B. napus* plants. (Das *et al.*, 2022) produced resynthesized *B. napus* by an interspecific cross between one genotype of each of *B. rapa* and *B. oleracea*: these resynthesized lines flowered and matured six days earlier than the check var- iety but not the early maturing *B. rapa* parent. Schranz and Osborn (2000) produced resynthesized *B. napus* from reciprocal interspecific crosses between single plants of *B. rapa* and *B. oleracea*, and observed stable and heritable variation for flowering time among advanced generation S_7 *B. napus* lines. The influence of flowering time regulators on many yield- related traits as well as on resistance to biotic and abi- otic stresses (Quijada *et al.*, 2006; Chen *et al.*, 2007; Basunanda *et al.*, 2010), makes flowering time a major driver of crop evolution, subject to strong selection during crop breeding (Schiessl *et al.*, 2014). Hence,

use of resynthesized lines has the potential to reintroduce useful diversity for this important trait.

F. Drought tolerance

Drought stress affects seed oil composition and decreases oil content, which is harmful to rapeseed quality and yield (Enjalbert *et al.*, 2013). Hatzig *et al.*, (2014) previously demonstrated that drought-tolerant and drought-susceptible cultivars of *B. napus* exhibit differential physiological responses related to abscisic acid and osmotic adjustment. However, limited gen- etic diversity in *B. napus* narrows the possibility to breed for drought tolerance and other environmental adaptation traits (Guo *et al.*, 2017). Therefore, breed- ing drought-resistant *B. napus* cultivars may require screening *B. rapa* and *B. oleracea* germplasm for drought tolerance traits. Guo *et al.* (2015) identified wide variation for drought tolerance in nine *B. rapa* accessions following physiological evaluation for drought responses under controlled environmental conditions. For example, a wild-type *B. rapa* ssp. *syl- vestris* was found to maintain mature plant biomass following an exposure to transient drought stress dur- ing the early seedling stage, compared to control con- ditions, whereas a cultivated *B. rapa* ssp. *trilocularis* suffered significant reductions in mature plant bio- mass under the same conditions (Guo *et al.*, 2015). Guo *et al.* (2017) identified biomarkers related to the drought resistance of *B. rapa*, which could be valuable for the breeding of resistant *B. napus* via interspecific crosses. Several studies have revealed a relationship between DNA methylation changes and drought toler- ance in important crops such as rice (Zheng *et al.*, 2013), maize (Wang *et al.*, 2021) and Faba bean (Abid *et al.*, 2017). For example, differences in drought tol- erance between different rice cultivars have been asso- ciated with heritable differences in DNA methylation levels (Wang *et al.*, 2011; Zheng *et al.*, 2013). Jiang *et al.* (2019) evaluated the physiological and methyla- tion changes in resynthesized *B. napus* lines in com- parison to its diploid *B. rapa* and *B. oleracea* parents, and observed an intermediate drought tolerance level between both parents. Drought tolerance in resynthe- sized *B. napus* is however still understudied, possibly because of the complexity of the mechanisms involved in plant response to abiotic stresses.

IV. Evolution of stable meiosis in allopolyploids

Chromosome pairing in allopolyploid meiosis occurs either as homologous or homoeologous pairing. Homologous pairing refers to preferential pairing between homologous partners (maternal and paternal chromosome pair) which then recombine to ensure exchange of genetic material (Rubin *et al.*, 2022) whereas homoeologous pairing refers to (nonpreferential partner) pairing between nonhomologues from two different subgenomes. Homoeologous pairing has been observed in nearly all allopolyploid species (Lloyd and Bomblies, 2016), but usually with low fre- quency in established allopolyploid species. Instead, nearly all established allopolyploid species show a dip- loid-like meiotic behavior at meiosis, indicating that precise control of homologous pairing is a prerequisite for polyploid species establishment (Pel,e *et al.* 2018) and confers evolutionary advantages in polyploid spe- cies (Jenczewski *et al.*, 2003). However, newly formed polyploids very commonly show irregular meiosis: specifically, chromosomes with high sequence similarity belonging to different subgenomes pair with one another (homoeologous recombination), thereby creating complex meiotic configurations (Ramsey and Schemske, 2002; Blasio *et al.*, 2022) leading to the production of unbalanced and aneuploid progeny (De Storme and Mason, 2014), chromosome rearrangements (Parkin *et al.*, 1995; Pires *et al.*, 2004; Udall *et al.*, 2005; Szadkowski *et al.*, 2010; Xiong *et al.*, 2011; Chalhoub *et al.*, 2014) and reduction in fertility (Xiong *et al.*, 2011a; Rousseau-Gueutin *et al.*, 2017; Ferreira de Carvalho *et al.*, 2021). Suppression of homoeologous pairing is critical for cytological dip- loidization to take place at meiosis in newly formed allopolyploids (Jenczewski *et al.*, 2003). Since homoe- ologous pairing is frequent in most newly formed allopolyploids, this implies that homologous pairing is ensured in established allopolyploids despite the pres- ence of homoeologous pairing partners. Cytological diploidization is the process by which the complex meiotic behavior of newly formed polyploids becomes "diploid-like," such that only homologous chromosome pairs form, thus producing genetically balanced gametes: this process is thought to be essential for allopolyploid speciation (Matsuoka *et al.*, 2014).

V. Discovery of meiotic instability in resynthesized *B. napus*

Frandsen (1947) was perhaps the first to observe abnormal meiosis in resynthesized rapeseed hybrids ($2n = 4x = 4$ AACC) formed between tetraploid × tetraploid *B. rapa* and *B. oleracea*. Heneen *et al.*, (2004) produced two cross combinations of resynthesized *B. napus* lines using two different varieties of *B. rapa*: *B. rapa* ssp.

oleifera var. yellow sarson, and a Swedish variety crossed with one *B. oleracea* var. *alboglabra* used as the maternal parent. A high frequency of both univalent and multivalent formation at metaphase as well as aberrant meiotic behavior at later stages was prevalent in the resynthesized line derived from the Swedish *B. rapa* compared to the line derived from the Indian yel- low sarson, which showed almost normal meiosis (Heneen *et al.*, 2004). These differences in meiotic behavior might be attributed to genetic factors that control homoeologous pairing (Heneen *et al.*, 2004), specifically the inheritance of different allelic variants of meiosis genes from the diploid *B. rapa* (*B. campest- ris*) Indian yellow sarson progenitor compared to the Swedish variety progenitor crossed to produce the resynthesized line. Genotypic differences were also found to play a major role in accumulation of copy number variants (putatively caused by differences in homoeologous recombination frequencies) in a larger set of resynthesized lines (Katche *et al.,* 2022), support- ing the hypothesis that inherited allelic variants of mei- osis genes determine genome stability in resynthesized rapeseed lines. Szadkowski *et al.* (2010) analyzed first generation resynthesized *Brassica napus* and observed frequent meiotic abnormalities, which were proposed to be the main drivers of genome instability.

Older studies on resynthesized *B. napus* using molecular markers have shown a higher frequency of homoeologous recombination in resynthesized lines compared to established *B. napus* cultivars (Parkin *et al.*, 1995; Sharpe *et al.*, 1995; Osborn *et al.*, 2003a; Udall *et al.*, 2005). Xiong *et al.* (2011) also detected no karyotype rearrangements in a *B. napus* cultivar using molecular cytogenetics, but found high frequen- cies of chromosome rearrangements in later genera- tions of resynthesized *B. napus* lines. Ferreira de Carvalho *et al.* (2021) evaluated genome stability and fertility in different resynthesized *B. napus* lines advanced by single seed descent with selection for euploidy to the S_9 generation, in contrasting genetic backgrounds, and observed a decrease in newly fixed homoeologous rearrangements. The effect of homoeol- ogous rearrangements on meiosis and seed fertility was also shown to be strongly dependent on genetic background and cytoplasm donor (maternal parent) (Ferreira de Carvalho *et al.*, 2021). Several candidate regions involved in seed yield and genome stability were identified from these rearranged homoeologous regions (Ferreira de Carvalho *et al.*, 2021). By con- trast, Rousseau-Gueutin *et al.*, (2017) analyzed 33 resynthesized *B. napus* individuals from two open pol- linated populations, and compared their meiotic behavior to the observed rearrangements but could not identify any clear correlation, possibly as a result of unknown genetic factors contributed by pollen donors during the open-pollination events. Katche *et al.*, (2022) evaluated 140 early and later generation resynthesized *B. napus* lines for inherited and novel copy number variants, and observed no novel CNVs in several later generation genotypes, indicating that these genotypes are putatively stable.

VI. Genetic control of homoeologous pairing in rapeseed

Specific genetic factors are thought to regulate pairing between homologous chromosomes and prevent homoeologous pairing from occurring (Jenczewski *et al.*, 2003). Such pairing control has already been reported in wheat (Sears, 1976; Lukaszewski and Kopecky, 2010; Mart,ın *et al.*, 2017; Rey *et al.*, 2017), tall fescue (Jauhar, 1975), oat (Rajhathy and Thomas, 1972; Thomas and Al-Ansari, 1988), and rapeseed (Jenczewski *et al.*, 2003; Jenczewski and Alix, 2004; Liu *et al.*, 2006). In bread wheat (*Triticum aestivum L.*: 2*n* ¼ 6*x* ¼ 42; AABBDD), several genetic loci responsible for the control of homologous recombin- ation have been identified (Riley and Chapman, 1958; Sears, 1976; Mart,ınez *et al.*, 2001). Of these, two major genetic loci *Pairing homoeologous 1* (*Ph1*; Riley and Chapman, 1958; Luo *et al.*, 1996) and *Pairing homoeologous 2* (*Ph2*; Sutton *et al.*, 2003; Serra *et al.*, 2021) which prevent or reduce homoeologous pairing have been identified. Studies on the structural and functional details of the wheat *Pairing homoeologous 1* (*Ph1*) locus in wheat and *Pairing regulator in B. napus* (*PrBn*) have provided important comparative insights into the origins and roles of those genes in the cyto- logical diploidization that occurred in the evolution of these two widely divergent taxa (Jenczewski *et al.*, 2003; Griffiths *et al.*, 2006; Al-Kaff *et al.*, 2008; Nicolas *et al.*, 2009).

The *PrBn* locus in rapeseed was discovered by Jenczewski *et al.*, (2003), who investigated a segregat- ing mapping population of 244 *B. napus* haploids (2*n* ¼ AC) produced from crosses between two estab- lished *Brassica napus* lines which showed different fre- quencies of homoeologous chromosome pairing as haploids (high pairing and low pairing). *PrBn* was identified by measuring and comparing the levels and distribution of the chromosome pairing between the A and C genomes of two *Brassica* allohaploids with contrasting chromosome pairing frequencies (Jenczewski *et al.*, 2003). Later, Liu *et al.*, (2006)

mapped this locus to chromosome *BnaC9* and identified several other minor loci with additive or epistatic effects. Nicolas *et al*., (2009) analyzed two diverse allohaploid *B. napus* progeny sets with different *PrBn* activity, and showed that the rate of recombination between both homologous and homoeologous chromosomes is affected by *PrBn* during meiosis depending on plant karyotype. However, the evaluation of the meiotic behavior of allotetraploid *B. napus* lines (Yudal and Darmor-*bzh*, 2*n* ¼ AACC) carrying different versions of the *PrBn* locus with different effects on homoeologous crossovers at Metaphase 1 revealed regular bivalent pairing and chromosome inheritance (Grandont *et al*., 2014), thereby undermining the role of this locus in regulating homoeologous recombin- ation in allotetraploid (2*n* ¼ AACC) *B. napus*. Therefore, it is still unclear whether *PrBn* controls homoeologous pairing in allotetraploid *B. napus*, as evidence for reduction of homoeologous pairing was only provided in *B. napus* allohaploids. More recently, Higgins *et al*., (2021) produced a segregating *B. napus* doubled haploid (DH) population from reciprocal crosses between spring-type *B. napus* and a resynthe- sized *B. napus* line, and identified a major QTL (32 − 58% effect) on *BnaA09* identified as *BnaPh1* (*B. napus Pairing homoeologous 1*) which contributed to the control of homoeologous recombination in *Brassica napus*. Several possible candidate genes were pro- posed, some of which will be discussed in detail in the subsequent section. Interestingly, the chromosome region *BnaC9* where *PrBn* was mapped to appears to be homoeologous to the *BnaA9* QTL region which was identified by Higgins *et al*., (2021).

Recently, Sourdille and Jenczewski, (2021) drew an analogy between genetic pairing control in wheat and tetraploid *B. napus*, reporting similarities in terms of the lower number of pairing homoeologous loci iden- tified in both wheat (*Ph1* and *Ph2*; Mart,ın *et al*., 2017; Serra *et al*., 2021) and *B. napus* (three QTLs including *BnaPh1*; Higgins *et al*., 2021). Sourdille and Jenczewski, (2021) also suggested two major differen- ces between the two systems. First, none of the puta- tive candidate genes identified within the *BnaPh1* loci (Higgins *et al*., 2021) function in the major crossover (CO) pathway (Class 1 COs), that has been implicated in homoeologous CO formation in *B. napus* (Gonzalo *et al*., 2019). In contrast, the *Ph1* locus in wheat that encodes *ZIP4* (Mart,ın *et al*., 2017) functions in the formation of COs (Pyatnitskaya *et al*., 2019). Second, the *BnaPh1* QTL region does not encode any identified candidate genes involved in the DNA mismatch repair system (Higgins *et al*., 2021), in comparison to the *Ph2* locus in wheat which has encodes *MSH7* (*MutS* homologue 7; Serra *et al*., 2021) that is involved in DNA damage recognition and repair as well as control of meiotic recombination (Culligan and Hays, 2000; Lario *et al*., 2015).

Several studies in neopolyploids have investigated the possibility that the genetic control of meiosis could be multifactorial. In synthetic wheat, the combined effect of both *ph1* and *ph2* mutants has been reported to promote homoeologous recombination (Ceoloni and Donini, 1993), suggesting that proteins encode in these genes found in the two loci could be interacting to prevent homoeologous recombination. Findings from (Martin *et al*., 2014; Mart,ın *et al*., 2017) showed that *ZIP4*, unlike *MMR* proteins, is not involved in promoting heteroduplex rejection following DNA-strand exchange between divergent sequences, suggesting that *ZIP4* and *MSH7* function differently, and that the mechanisms controlling homoeologous recombination could be diverse. In autotetraploid *Arabidopsis arenosa*, at least eight meiosis-related genes have been shown to be under strong selection, indicating that meiotic adaptation in this lineage is polygenic (Hollister *et al*., 2012; Yant *et al*., 2013) and could involve other interacting protein partners (Morgan *et al*., 2022). Several potential ways to achieving meiotic stabilization in neopolyploids have been reviewed by Gonzalo, (2022). Although many of these routes have been investigated so far only in newly synthesized autopolyploid *Arabidopsis*, a few have already been investigated in newly synthesized *B. napus*. However, more studies still need to be carried out in order to come to a strong conclusion on the different routes to meiotic adaptation in allopolyploids (and in *B. napus* specifically).

Of particular interest for means via which allopolyploid stabilization may occur is the idea that preexisting allelic variants present at unknown frequencies in the lower-ploidy progenitor species might act to stabilize meiosis in the neopolyploid, and the competing idea is that evolutionary selection for de novo mutations occurring after allopolyploid formation may confer meiotic stabilization. Samans *et al*., (2017) detected de novo allelic variants in natural and synthetic *B. napus* which are potentially involved in meiotic stabilization. Szadkowski *et al*. (2010) observed that meiosis was more stable in some synthetic *B. napus* genotypes analyzed compared to others, and suggested that this could be dependent on inherited allelic variants from the diploid progenitor. Supporting the role of novel mutations following polyploid establishment in restoring meiotic stability is genome fractionation, a process by which

duplicated genes in an allopolyploid return to single copy (Langham *et al.*, 2004). Genes which are involved in meiosis and DNA repair have been shown to be among the most rapidly returned to single copy (De Smet *et al.*, 2013; Lloyd *et al.*, 2014), supporting the idea that gene dosage (number of functional copies of each meiosis gene) may play a role in meiotic stabilization. Gonzalo *et al.*, (2019) observed that knocking out one of the copies of the *MSH4* gene in *B. napus* prevents homoeologous recombination, suggesting that fraction- ation of certain meiosis-related genes might be relevant for meiotic adaptation. Epigenetic regulation may also have a role to play in the adaptation of meiosis in allopo- lyploids (Gonzalo, 2022), as suggested by recent studies in both natural and synthetic allotetraploid *A. suecica* (TTAA) and *B. napus* (Jiang *et al.*, 2021; Yin *et al.*, 2021). Yin *et al.*, (2021) analyzed natural and synthetic *B. napus*, and demonstrated that DNA methylation and expression levels of meiosis-related and DNA repair genes including *MSH6*, which encodes a DNA mismatch repair protein, were significantly downregulated in synthetic *B. napus*. Similarly, Jiang *et al.*, (2021) analyzed the gene ontology (GO) enrichment of meiosis-related and differentially methylated genes in synthetic and natural allotetraploid *A. suecica* derived from the hybridization of *A. thaliana* (TT) and *A. arenosa* (AA), and found DNA methylation levels in three meiosis-related genes (*SMC1*, *SMC6B*, and *PDS5B* were lower in the F_{10} synthetics compared to natural *A. suecica* (Jiang *et al.*, 2021). These studies seem to suggest that meiotic stabilization could be affected by methylation levels in synthetic allopolyploids. Advanced sequencing and epigenetic tools as well as knock-out studies for more putative meiosis-related genes are necessary in the future to fully understand the evolution of meiotic stability, and to artificially recreate genomically stable synthetic allopolyploids.

VII. Meiosis genes implicated in genome stability in *Brassica napus* and resynthesized lines

A gradual improvement of meiotic stabilization over evolutionary time might have been necessary for the adapted meiosis observed in established polyploids (Lloyd *et al.*, 2014), possibly also in conjunction with more dramatic early mutations or inherited allelic variants. One potential way to that meiosis in neopolyploids may have adapted is through loss of functional gene copies (Gonzalo *et al.*, 2019). Genes involved in meiotic recombination have been shown to rapidly return to a single copy, faster than the genome-wide average for all genes following a polyploidisation event (Lloyd *et al.*, 2014). The process by which these duplicated gene copies are lost is known as genome fractionation (Langham *et al.*, 2004). Gonzalo *et al.*, (2019) analyzed *Brassica napus* allohaploids to deter- mine the effect of copy number reduction of *MSH4* on crossover formation. Nonhomologous crossovers were shown to originate almost exclusively from the *MSH4*-dependent pathway, and decreased in number when *MSH4* returned to single copy (homologous crossovers were unaffected) (Gonzalo *et al.*, 2019). *FANCM* (Fanconi Anemia Complementation Group M) is another protein which has been shown to be involved in meiotic recombination by promoting non- crossover activity through the *SDSA* (synthesis- dependent strand annealing) pathway in *Brassica* species (Crismani *et al.*, 2012). Blary *et al.* (2018) pro- duced segregating populations of 20−140 *Brassica napus* allohaploid plants derived from two double A/C *fancm* mutant F_1 plants and four F_1 hybrids as well as two wild-type siblings. *FANCM* limits homolo- gous crossovers and crossover formation in *Brassica napus* (euploids) and allohaploids respectively, and is present in a single copy per *Brassica* genome, although *fancm* mutants show an increase in homoe- ologous crossovers in allohaploids (Blary *et al.*, 2018). The anti-crossover activity of *FANCM* was shown to be conserved across *Brassica* species (Blary *et al.*, 2018). Higgins *et al.* (2021) identified 12 meiotic can- didate genes suggested from three QTL which contrib- uted to the control of homoeologous recombination. Two interesting candidates out of five genes underly- ing the major *BnaA9* QTL region were *RPA1C* (Replication Protein A 1 C) and *MUS81* (*MMS* and UV Sensitive 81) (Higgins *et al.*, 2021). *RPA1C* func- tions in double-strand-break repair in early meiosis in *A. thaliana* (Aklilu *et al.*, 2014) whereas *MUS81* is also a DNA repair protein which is implicated in the *ZMM*-independent crossover pathway (Berchowitz *et al.*, 2007; Higgins *et al.*, 2008). Another interesting candidate gene out of seven meiosis genes identified in one of the other two minor-effect QTL was *MSH3*, which is one homologue of the *MutS* gene, a major controller of mismatch repair in *E. coli* (Kunkel and Erie, 2005). Similarly, Samans *et al.*, (2017) analyzed short-read resequencing data to compare between natural and synthetic *Brassica napus*, and identified 17 genes implicated in the meiotic mismatch repair system, including orthologs of *MSH2*, *RAD51b*, and *NAP1* as putative candidate genes of interest for control of homoeologous recombination. Gaebelein *et al.*, (2019) identified three putative candidate meiotic

genes (*RAD51*, *SMC5*, and *SYN4/RAD21.3*) underlying QTL for fertility in a translocated region between chromosome A03 and C03 in the *Brassica napus* parent donor used to produce a *Brassica* allohexaploid population (2*n* ¼ 6*x* ¼ AABBCC) derived from the cross *B. napus* (AACC) × *B. carinata* (BBCC) × *B. juncea* (AABB), followed by two or three generations of self-pollination. Xin *et al.*, (2016) also found via investigation of a male-sterile *B. napus* mutant that *MS5* participates in progression of meiosis during early prophase I and that its allelic variants lead to differences in fertility. *MLH1* has been implicated in the increase in crossover formation in *Brassica* allote- traploid hybrids compared to its diploid parents (Leflon *et al.*, 2010).

Increased crossover formation is a general feature of newly formed allopolyploids and may be necessary for their establishment (Grandont *et al.*, 2014). Hence, there are potential benefits associated with increased crossover formation between homologues (Schiessl *et al.*, 2019). Synthetic allotetraploids show an increase in crossover rate (which becomes extreme in allotri- ploids; Leflon *et al.* 2010) that can be exploited for introgression breeding programs (Serra *et al.* 2021). However, the suppression of crossover formation between homoeologues is probably necessary for allo- polyploid speciation (Grandont *et al.*, 2014).The identification of genes such as *FANCM* which suppress homoeologous crossovers (Crismani *et al.*, 2012; Blary *et al.*, 2018), suggest fruitful avenues for investigation of how the suppression of homoeologous recombin- ation could lead to the stabilization of meiosis in newly formed polyploids.

VIII. Novel genetic and genomic changes in resynthesized *Brassica napus*

A. Proteomic and gene expression changes

Newly formed allopolyploids and synthetic polyploids often undergo extensive and rapid genome changes within the first generations following whole genome duplication (WGD) (Adams and Wendel, 2005) including sequence rearrangements, homoeologous recombination, sequence elimination, and changes in DNA methylation (Liu and Wendel, 2003; Osborn *et al.*, 2003b; Levy and Feldman, 2004; Lukens *et al.*, 2006; Gaeta *et al.*, 2007; Szadkowski *et al.*, 2010). Interspecific hybridization and polyploidization to produce resynthesized and natural allopolyploids results in genomic shock, which includes not only the above mentioned effects but also changes in gene expression (Albertin *et al.*, 2009; Gaeta *et al.*, 2009; Jiang *et al.*, 2013; Zhang *et al.*, 2016), epigenetic changes (Matzke *et al.*, 1999; Levy and Feldman, 2004; Xu *et al.*, 2009), transposon activation (Kashkush *et al.*, 2002; Kantama *et al.*, 2013; Sarilar *et al.*, 2013), and transcriptomic changes (Fu *et al.*, 2016; Palacios *et al.*, 2019; Wei *et al.*, 2019). These genomic changes could potentially produce new gene complexes, and promote rapid evolution (Soltis and Soltis, 1999). Polyploid species incorporate genetic variation from their genetically diverse diploid progenitors, thereby maintaining a high level of genetic variation (Soltis and Soltis, 1995; Brochmann *et al.*, 1998; Cook *et al.*, 1998; Segraves *et al.*, 1999), which can be exploited for breeding and research.

Polyploidy is also known to broadly affect gene expression, and particularly to lead to gene silencing (Liu and Wendel, 2003; Osborn *et al.*, 2003b). Genes that are duplicated by polyploidy may be subsequently expressed at equal levels, or there could be unequal expression or silencing of one gene copy (Adams and Wendel, 2005). Silencing can occur as early as the first generation following polyploidisation, while some genes are not silenced until later generations (Wang *et al.*, 2004). Several studies have analyzed changes in gene expression in order to provide evidence for gene silencing, additive or nonadditive gene expressions as well as alterations in DNA methylation patterns. Albertin *et al.* (2006) analyzed gene expression in four different newly synthesized *B. napus* lines as well as their diploid *B. rapa* and *B. oleracea* parents, and observed gene silencing as well as changes in expres- sion patterns. Resynthesized *B. napus* lines displayed proteomic patterns slightly closer to the *B. rapa* pater- nal genome donor compared to *B. oleracea* in both stem and root tissues (Albertin *et al.*, 2006). The pre- dominant gene expression pattern was of paternal ori- gin, with no bias in the cellular localization of proteins displaying nonadditive values (Albertin *et al.*, 2006). Gaeta *et al.* (2009) analyzed three independ- ently-derived resynthesized *B. napus* lineages, and observed that only a limited number of genes show nonadditive gene expression while most were addi- tively expressed. Kong *et al.* (2011) observed extensive modification of leaf proteomes in resynthesized
B. napus lines, although there was no disturbance of housekeeping genes.

Wang *et al.* (2004) analyzed differentially expressed genes in S_1 to S_4 generations of synthetic *A. suecica* (2*n* ¼ 4*x* ¼ 26) produced by hybridization between autotetraploid *A. thaliana* (2*n* ¼ 4*x* ¼ 20) and *A. arenosa* (2*n* ¼ 4*x* ¼ 32) as well as natural *A. suecica* and their diploid and autotetraploid progenitors, and

observed significant numbers of differentially expressed genes in the newly formed polyploids rela- tive to their established parent species. Novel gene expression was also observed, and some genes expressed in the parents were silenced in the early generation progeny (S_1 to S_3) while other genes were silenced in later generations (Wang *et al.*, 2004). By contrast, Yoo *et al.*, (2013) investigated gene expres- sion patterns in interspecific F_1 hybrid, synthetic and natural allopolyploid cotton (AADD) as well as their diploid progenitors *G. arboretum* (AA) and *G. rai- mondii* (DD) by RNA sequencing of the leaf tissues. They found natural allopolyploid cotton had a greater fraction of transgressive and novel gene expression patterns between subgenome homoeologs relative to the diploid parents than the newly formed synthetics (Yoo *et al.*, 2013). These studies suggest that gene expression changes following polyploidy may not necessarily follow similar or predictable patterns between taxa, or even between lineages within taxa, which may also explain the somewhat contradictory results found so far for synthetic *B. napus*.

B. DNA methylation changes

Apart from proteomics and gene expression studies, resynthesized *B. napus* lines have also been investigated for changes in DNA methylation. Song *et al.*, (1995) first detected DNA methylation changes in resynthesized *B. napus* lines. Lukens *et al.*, (2006) analyzed 49 early generation resynthesized *B. napus* lines, and observed that genetic changes were rare but cytosine methylation changes were frequent. Gaeta *et al.*, (2007) analyzed the same population of resynthesized *B. napus* at S_0 and S_5 generations, and found that most of the methylation changes observed in the S_0 generation remained fixed in the S_5 generation, although a small proportion reverted and some new changes were observed. Gaeta *et al.* (2007) observed that although genetic changes in S_5 CCAA resynthe- sized *B. napus* occurred more frequently in the C sub- genome, cytosine methylation changes occurred more frequently in the A subgenome. Yin *et al.* (2021) ana- lyzed DNA methylation patterns in both late gener- ation synthetic (F_{12}) and natural *B. napus*, and observed significantly higher methylation levels (espe- cially CHG) in the synthetics. Gene ontology analysis showed significant down-regulation of differentially methylated regions enriched for meiosis genes in the synthetics compared to in natural *B. napus*, suggesting possible correlations between DNA methylation and genome stability (Yin *et al.*, 2021). In *A. suecica* (TTAA), lower CG methylation levels were observed in both F_1 and F_{10} generations of resynthesized as well as natural lines, particularly in the A subgenome (Jiang *et al.*, 2021). This study also observed down- regulation of differentially methylated regions enriched for meiosis genes in synthetic compared to natural *A. suecica* (Jiang *et al.*, 2021). Song *et al.*, (2017) investigated methylation changes in resynthe- sized cotton ($2n = 4x = 52$; AADD) hybridized between *Gossypium arboretum* ($2n = 2x = 26$; AA) and *Gossypium raimondii* ($2n = 2x = 26$; DD) as well as natural cotton in both cultivated and wild forms. They detected lower CHH methylation levels in the resynthesized cotton compared to parents, although CG and CHG methylation levels were similar in both.

CG methylations levels were found to be higher in the A compared to the D subgenome homeologues in tetraploid cotton (Song *et al.*, 2017). Ran *et al.*, (2016) observed increased DNA methylation levels in S_0–S_3 resynthesized *B. napus* lines, with the lowest methylation levels detected in the S_0. The *B. oleracea* parent also showed higher methylation levels compared with the *B. rapa* parent. However, gene expression in resynthesized *B. napus* and its diploid parents is inconsistent with observed methylation patterns (Ran *et al.*, 2016), as has also been observed in established *Brassica* species (Liu *et al.*, 2014; Parkin *et al.*, 2014). Similarly, only a small correlation between methyla- tion and gene expression have been shown in *Brassica oleracea* (Parkin *et al.*, 2014).

C. Subgenome dominance and homoeologous gene expression bias

Subgenome dominance is defined as the phenomenon by which one subgenome retains more genes, and by which gene copies in one subgenome are more highly expressed than their copies in the other subgenome/s (Cheng *et al.*, 2016a). More specifically, homoeolog expression bias refers to the preferential expression of one homoeolog relative to the other (Grover *et al.*, 2012), and this phenomenon may be coupled with biased fractionation, which is the preferential loss of gene copies from one of two or more divergent subgenomes following whole genome duplication (Wendel *et al.*, 2018). Subgenome dominance has been observed in many allopolyploids, although not in all allopolyploids (and not in any autopolyploid) (Zhao *et al.*, 2017; Bird *et al.*, 2018).

Previous studies have shown that one subgenome in an allopolyploid often retains significantly more genes compared to the other subgenomes (Bird *et al.*,

2018). Most of the genes which were preferentially retained in *Arabidopsis* arising from the most recent whole genome duplication originated from one (dominant) subgenome compared to the other (recessive) subgenome (Thomas *et al.*, 2006). By contrast, Burns *et al.*, (2021) found no evidence of homoeologous gene expression bias in synthetic or natural *A. suecica* relative to their diploid progenitors: one subgenome did not contribute more than the other to homoeolo- gous gene expression and homoeologous gene pairs were highly correlated in expression across tissues (Burns *et al.*, 2021). In cotton, homoeologous gene expression was found to be balanced in both interspe- cific F_1 hybrid (AD) and natural allopolyploids (AADD), while the synthetic allopolyploid (AADD) showed a bias toward the A over the D subgenome (Yoo *et al.*, 2013). In *Mimulus peregrinus* (2*n* ¼ 6*x* ¼ 92) hybridized from the cross between *Mimulus luteus* (2*n* ¼ 4*x* ¼ 60–62) and *Mimulus gutatus* (2*n* ¼ 2*x* ¼ 28), genes from the dominant subgenome have significantly higher expression, and this pattern was probably established in the first generation and increased over subsequent generations in the observed resynthesized hybrids (Edger *et al.*, 2017). However, subgenome-wide expression bias is not found in all plant species. For instance, in *B. napus* (Chalhoub *et al.*, 2014; Bird *et al.*, 2021), wheat (Pfeifer *et al.*, 2014; Harper *et al.*, 2016) and cotton (Yoo *et al.*, 2013), global gene expression is not biased toward one specific subgenome. Homoeologous gene expression observed in *B. napus* showed no pattern of significant bias toward either the A or C subgenome, similar to older studies (Chalhoub *et al.*, 2014). However, local regions favoring one subgenome over the other have been observed (Chalhoub *et al.*, 2014; Bird *et al.*, 2018). Bird *et al.*, (2021) analyzed six resynthesized *B. napus* (CCAA) lines, and observed a significant expression bias toward the maternal *B. oleracea* subge- nome with approximately 70% of biased homoeolog pairs showing the same bias relationship in all six lines and in the parents. By contrast, Ferreira de Carvalho *et al.*, (2019) produced 225 plants across three generations obtained from two crosses between different accessions of *B. oleracea* × *B. rapa*, and observed that resynthesized *B. napus* showed no biased subgenome expression. This buttresses the claim that subgenome expression bias dominance may be predominantly inherited from progenitors rather than an outcome of interspecific hybridization and whole genome duplication (Buggs *et al.*, 2014). In established *B. napus* (Chalhoub *et al.*, 2014) as well as other *Brassica* hybrid types with the A and C genomes (Gaebelein *et al.*, 2019; Katche *et al.*, 2021), significant subgenome bias has also been observed following homoeologous exchanges, where more A to C homoeologous subgenome replacements are found compared to C to A replacements. However, how or if this relates to biased fractionation or gene expression bias between subgenomes is as yet unknown (Mason and Wendel, 2020): although recent findings seem to sug- gest that biased fractionation or gene expression bias might be related to these subgenome replacements, no strong conclusion has yet been reached.

IX. Conclusions

Resynthesized *B. napus* has long been considered genomically unstable, and hence unsuitable as direct breeding material. However, recent studies have shown that some resynthesized *B. napus* lines are genomically stable and fertile after a few rounds of self-pollination, and hence may also be stably main- tained for many generations as resources for research and breeding. Further investigation is still needed to understand the genetic factors underlying genome sta- bility differences between genotypes. In breeding, resynthesized *B. napus* has been utilized for the intro- gression of agronomically important traits into com- mercial rapeseed cultivars, as well as for the experimental study of many other potentially useful traits. However, traits such as herbicide resistance, oil quality, insect and nematode resistances as well as abi- otic stresses such as drought, cold, and salt tolerance are areas where little or no research has been done. These traits in resynthesized rapeseed should be inves- tigated further for future crop improvement. Nevertheless, many resynthesized rapeseed lines still possess poor agronomical traits such as low seed yield and oil content, high glucosinolate and high erucic acid content, and reduced winter hardiness. To over- come these challenges, semi-resynthesized *B. napus* lines with better agronomic performance can be pro- duced by crossing resynthesized *B. napus* lines with elite rapeseed cultivars. Resynthesized rapeseed remains an indispensable genetic resource for the broadening of genetic diversity in rapeseed as well as for the introgression of specific agronomic traits, as well as an interesting system to investigate meiotic stabilization and genome change in newly-formed allopolyploids.

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3.0 Fertility, genome stability, and homozygosity in a diverse set of resynthesized rapeseed lines

Elizabeth Ihien Katche, Antje Schierholt, Heiko C. Becker HC, Jacqueline Batley, Annaliese S. Mason

3.1 Publication Outline

The following publication describes the purity (homozygosity), fertility, and genome stability of resynthesized *B. napus* lines produced by crosses between *B. rapa* and *B. oleracea*, as well as between *B. rapa* and wild C genome species. Most of the lines which have been propagated over multiple generation were found to be heterozygous: contaminated as a result of outcrossing with unknown parents. Fertility and genome stability were both genotype-dependent. A high number of copy number variants (CNVs) were observed in most lines, which was significantly associated with reduced fertility. Eight putatively stable *B. napus* lines were detected.

3.2 Publication

Authors Contribution

Elizabeth Ihien Katche: Methodology, Investigation, Writing- original draft. Antje Schierholt: Resources, Writing- review and editing. Heiko C. Becker: Resources, Writing- review and editing. Jacqueline Batley: Methodology, Writing- review and editing. Annaliese Mason: Conceptualization, Funding acquisition, Super- vision, Writing- review and editing.

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Fertility, genome stability, and homozygosity in a diverse set of resynthesized rapeseed lines

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abstract

Rapeseed (*Brassica napus*, AACC) was formed by hybridization between progenitor species *Brassica rapa* (AA) and *Brassica oleracea* (CC). As a result of a limited number of hybridization events between specific progenitor genotypes and strong breeding selection for oil quality traits, rapeseed has limited genetic diversity. The production of resynthesized *B. napus* lines via interspecific hybridization of the diploid progenitor species *B. rapa* and *B. oleracea* is one possible way to increase genetic variation in rapeseed. However, most resynthesized lines produced so far have been reported to be meiotically unstable and infertile, in contrast to established *B. napus* cultivars. This hinders both maintenance and use of this germplasm in breeding programs. We characterized a large set of 140 resynthesized lines produced by crosses between *B. rapa* and *B. oleracea*, as well as between *B. rapa* and wild C genome species (*B. incana*, *B. hilarionis*, *B. montana*, *B. Bourgeaui*, *B. villosa* and *B. cretica*) for purity (homozygosity), fertility, and genome stability. Self-pollinated seed set, seeds per ten pods as well as percentage pollen viability were used to estimate fertility. SNP genotyping was performed using the Illumina Infinium *Brassica* 60K array for 116 genotypes, with at least three individuals per line. Most of the material which had been advanced through multiple generations was no longer pure, with heterozygosity detected corresponding to unknown parental contributions via outcrossing. Fertility and genome stability were both genotype- dependent. Most lines had high numbers of copy number variants (CNVs), indicative of meiotic instabil- ity, and high numbers of CNVs were significantly associated with reduced fertility. Eight putatively stable resynthesized *B. napus* lines were observed. Further investigation of these lines may reveal the mecha- nisms underlying this effect. Our results suggest that selection of stable resynthesized lines for breeding purposes is possible.

1. Introduction

Brassica napus (rapeseed, 2*n* = AACC) is a relatively young species spontaneously formed by hybridization between *Brassica rapa* (AA) and *Brassica oleracea* (CC) in the last 10,000 years [1]. It belongs to the Brassicaceae family and is a member of the U's Tri- angle species [2], which comprise three diploids with genomes AA, BB and CC (*B. rapa*, *B. nigra* and *B. oleracea*) and three allote- traploids with genomes AABB, AACC and BBCC (*B. juncea*, *B. napus* and *B. carinata*). Rapeseed is an economically important oil crop globally, and many breeding strategies have been employed to improve its different agronomically useful traits. However, the genetic diversity of established rapeseed cultivars is limited as a result of few hybridization events between *B. rapa* and *B. oleracea* as well as intensive selection for high quality rapeseed oil with low glucosinolate and zero erucic acid content [3]. Resynthesized *Brassica napus* lines are potentially useful resources in expanding the limited genetic diversity of established rapeseed cultivars [4,5]. Resynthesized *B. napus* has been studied for the improvement of rapeseed for agronomically useful traits such as disease resistance [6–8], pod shatter resistance [9,10], drought stress [11], yield [12–14], insect resistance [15–17], yellow seededness [18], and flowering time [19–22].

Several studies have produced resynthesized *B. napus* lines via interspecific crosses between diploid progenitor species of *B. rapa*

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and *B. oleracea* to produce F_1 hybrids (AC) followed by treatment with colchicine to produce tetraploid resynthesized *B. napus* (AACC) plants [4,12,23–30]. Karim et al. [12] crossed two different genotypes of *B. rapa* and *B. oleracea* cultivars in five reciprocal cross combinations and produced 17 resynthesized *B. napus* F_3 plants with 2 to 4.6 times higher yield, as well as increased number of pods per plant, compared to adapted short duration *B. napus* culti- vars. Malek et al. [29] also produced resynthesized *B. napus* lines by a cross between one genotype of each of *B. rapa* and *B. oleracea* which were higher in pollen fertility, 1000-seed weight, and higher mean seed yield per plant than both parents. Wild C genome spe- cies (*B. incana*, *B. hilarionis*, *B. montana*, *B. Bourgeaui*, *B. villosa* and *B. cretica*), instead of vegetable-type *B oleracea,* were also crossed with *B. rapa* genotypes to produce resynthesized *B. napus*. How- ever, low seed set and very poor winter hardiness were observed in most of the resynthesized lines with a wild C genome parent, although the lines produced high yielding hybrids when crossed with adapted genotypes of established *B. napus* cultivars [24].

To date, although several studies have produced synthetic *Brassica* hybrids ([4,12,24,31–33], all lines produced have been meiotically unstable, with frequent non-homologous pairing between chromosomes belonging to the A and C subgenomes [34,35]. Abnormal phenotypes such as off-type morphological characters, lower pollen viability, and lower seed set in resynthesized *B. napus* lines have been linked to aberrant meiosis [36] as well as generally poor fertility, and a number of sterile or nearly sterile resynthesized lines have been reported [37,38], although established *B. napus* cultivars are meiotically stable. Two hypotheses have been proposed to explain why the established *Brassica napus* species is meiotically stable and the resynthesized lines are unstable. One hypothesis is that established *B. napus* might have gained genetic control via inheritance of allelic variants from its diploid progenitors [39]. The second hypothesis proposes that a novel mutation in newly resynthesized *B. napus* after the initial hybridization event established meiotic stability [40]. Several other studies support this second hypothesis by suggesting that new mutations in terms of different kinds of genomic changes could potentially play a role in the establishment of a stable meiosis in natural *B. napus* [32,41–45]. However, only a few fixed chromosomal rearrangements caused by recombination between homoeologous regions of A and C genomes have been identified in *B. napus* cultivars [44,46,47], suggesting widespread chromosome rearrangement due to genome instability probably did not occur in the early generations after *B. napus* formation. In contrast, studies in resynthesized *B. napus* show frequent chromosome exchanges between the A and C subgenomes leading to extensive chromosome rearrangements and structural variants [23,25,32,48]. Recently, we also identified allelic variants present in parent diploid progenitor genotypes that contributed to different frequencies of non-homologous recombination events in first-generation resynthesized lines [39].

Resynthesized *B. napus* lines can still be viable for many generations despite genome instability and abnormal meiosis [48]. However, a few studies have observed drastic reduction in fertility of resynthesized *B. napus* lines across subsequent generations of self-pollination [23,27,48], thereby limiting the potential utilization of these lines for commercial breeding purposes. Other studies in *Brassica* resynthesized lines have demonstrated increased fertility over subsequent generations [49–51]. Although these differences may be due to genotype-specific effects as few genotypes of resynthe- sized lines have been investigated to date. In this study we aimed to determine genotypic differences in fertility and genome stability and to assess purity in a large set of resynthesized *B. napus* lines propagated over many generations via self-pollination.

We investigated a large set of 140 synthetic *B. napus* genotypes which had been propagated over several generations for homozygosity, fertility, and genome stability. Our investigations revealed only 33 lines with at least two individuals which were putatively free of cross-contamination via outcrossing to unknown parents. These lines were used to determine genotype-specific effects on genome stability as measured by presence of large-scale copy number variants (CNV) which usually result from unstable meiosis in synthetic *Brassica* hybrids [52].

2. Materials and methods

2.1. Plant materials and growth conditions

The materials used in this study were mostly derived from two distinct synthetic *Brassica napus* groups received from Georg August University Goettingen, Germany (File S1). The first group, referred to as "domesticated resynthesized *B. napus* lines" [4] were derived from hybridization between domesticated vegetable-type *B. rapa* and *B. oleracea* parents, and inherited a "winter-type" vernalization requirement from at least one parent (Fig. 1A; File S1). The second group, referred to as "wild C genome species resynthesized lines" produced by [24] also required vernalization (Fig. 1B; File S1). The parents of these lines were mostly sourced from germplasm banks such as Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben Germany (for cultivars), the Centre for Genetic Resources, the Netherlands (CGN), and the Germplasm Bank of the Higher Technical School of Agricultural Engineering of Madrid, Spain (for wild species). An extra six domesticated winter-type resynthesized lines produced by [6] at Justus Liebig University were also used in this study. The domesticated resynthesized group mostly have codes starting with S, G, K, L, and R, while the wild C genome group lines start with the code "J" or three letters, like INY or MOL, which indicate the cross combination (*B. incana* crossed with yellow sarson = INY, for example). The origin and production of these resynthesized lines has been previously described in detail [4,13,24].

In total, 140 genotypes of synthetic *B. napus* lines were used in this study. The lines comprised 121 domesticated resynthesized *B. napus* lines, and 19 wild C-genome species resynthesized lines.

At least three seeds from each of the 140 genotypes were sown in quick-pots and seedlings transferred to small pots between September and November 2017 under glasshouse conditions at Justus Liebig University. *Brassica rapa* and *B. oleracea* parent controls, including *B. napus* cultivars, were also sown. Genotypes with less than ten seeds as well as very old seeds stored under dry storage conditions were cultured on agar. Germinated seedlings were vernalized at a temperature of 4 °C for 10 and 12 weeks respec- tively at 4 to 6 leaf stages. The plants were then transferred back to the glasshouse after vernalization where they were bagged at flowering to allow self-pollination to occur. Resynthesized lines which survived vernalization and produced seeds after self- pollination (Table S1) were used for further analysis.

2.2. SNP genotyping using the Illumina Infinium 60K Brassica SNP array and subsequent cleaning and filtering of SNP data

Leaf samples were collected in 2 mL micro-centrifuge tubes at the 4 to 6 leaf stages, shortly before vernalization, and stored at −20 °C until use. DNA was extracted for 390 individual resynthesized plants (116 genotypes) using the BioSprint 96 plant work station (Qiagen, Hilden, Germany) according to the manufacturer's instruction (https://qiagen.com/). Single nucleotide polymorphism (SNP) genotyping was carried out using the high-throughput Illumina Infinium 60K *Brassica* SNP array for the resynthesized lines. Hybridization protocols were performed according to the manufacturer's instructions for all samples. SNP data were analysed, visual-

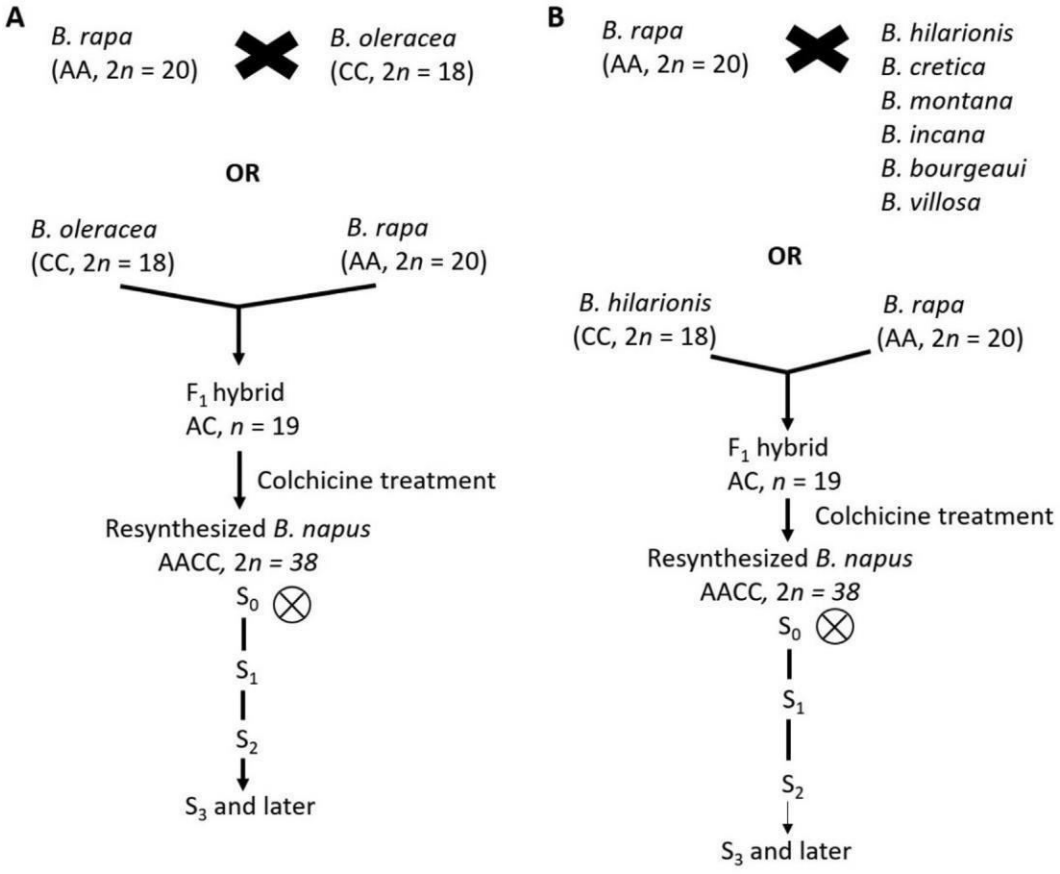

Fig. 1. Production and crosses between different groups of resynthesized *Brassica napus* lines. (A) The domesticated resynthesized *Brassica napus* lines produced from crosses between different *B. rapa* and *B. oleracea* parent species. (B) *Brassica napus* resynthesized lines produced from crosses between the wild C genome species *B. hilarionis*, *B. cretica*, *B. montana*, *B. incana*, *B. bourgeaui* and *B. villosa* with *B. rapa*.

ized, and exported into text files using 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 genome after application of the recommended brassica 60K cluster file [53]. Subsequently, A- and C-genome SNP probe sequences were mapped to the reference gen- ome of *B. napus* Darmor-*bzh* version 8.1 [54], using the top BLAST hit (Altemeyer). SNP filtering was performed as follows: (1) all unmapped SNPs and SNPs mapping to unplaced contigs were removed. (2) SNPs which mapped to the A genome but which amplified in *Brassica carinata* (BBCC) and *Brassica oleracea* (CC) control samples, as well as SNPs which mapped to the C genome but amplified in *Brassica juncea* (AABB) and *Brassica rapa* (AA) control samples, were filtered out. SNP markers across rows with missing calls (2 25%) and (2 166) "no call" counts as well as SNP markers which still showed > 60% heterozygous calls (AB) after removal of SNPs that were heterozygous in five doubled-haploid *B. napus* cultivars (CBWA Boomer, Monty_028DH, Surpass400_024DH, CBWA_Trilogy and Westar_10DH) were removed. After filtering, 21,938 SNPs were retained: 8369 SNPs in the A genome and 13,569 SNPs in the C genome (File S2). Genotype calls were then converted to homozygous/heterozygous calls (0 and 2 for homozygous and 1 for heterozygous) and incidence of missing calls represented by NA.

2.3. Assessment of purity in resynthesized B. napus lines

The purity of 116 resynthesized lines with SNP genotyping information was assessed after quality filtering of SNPs. Resynthesized lines which were produced by double haploid parental crosses between *B. rapa* and *B. oleracea* (AA x CC) are expected to be completely homozygous. Purity was assessed using three criteria: (1) the absence of continuous blocks of heterozygous (AB) calls across the A and the C genome in all individuals; (2) percentage heterozygosity in AB calls < 0.4% in resynthesized individuals across SNPs between the A and C genomes; and (3) screening of dendrogram plots produced separately for A and C genome SNP markers for all individuals to detect any contamination due to outcrossing of resynthesized lines with either natural *B. napus* or any other species on the field. All lines which deviated from these criteria were regarded as "putatively contaminated lines" (File S3), due to outcrossing or labelling mishaps, while the others which fulfilled all criteria are "putatively pure lines" (File S4). The phylogenetic relationship between all the resynthesized lines was plotted for the A and C genomes separately (Figs. S1, S2).

2.4. Fertility assessment in resynthesized B. napus lines

Fertility was assessed in 131 resynthesized lines with available fertility data using the total number of self-pollinated seeds, seeds per ten pods, and percentage pollen viability as measures of fertility. In order to assess percentage pollen viability in resynthesized lines, two freshly opened flowers were collected per plant and pollen grains stained with 1%–2% aceto-carmine solution [55]. At least 600 pollen grains per plant were counted and pollen viability was assessed using the Leica microscope (Leica DMR, Leica Microsystems). The plants were bagged to ensure self-fertilization and total self-pollinated seed-set as well as seed per ten pods were counted for each plant after harvesting.

2.5. Detection of copy number variation (CNV) in resynthesized B. napus lines

We assessed copy number variation (CNV) for the whole set of lines used in this study with SNP genotyping information (File S5) using the LogR ratio, which is an output metric of GenomeStudio that can be used to estimate allele copy number [50,56]. Out of 39 putatively pure resynthesized genotypes, we selected 33 lines with SNP genotyping data for at least two individuals. We estimated novel and inherited CNV in the putatively pure set of 33 lines (102 plants) by manually scoring CNV as deletion, reduced copy, and higher copy (Fig. 2) using the logR ratio, with estimated cut off values to score the type of CNVs in all three individuals of the same line. LogR ratios used were above 0.2 for higher copy number (one or 2 additional copies), values from −0.2 to −0.5 for reduced copy number (one missing copy), and below −2 for deletion (2 missing copies), according to previously established empirical values [56]. Only regions which were > 1 Mb in size were considered. "Novel" copy number variants were assumed (resulting from the previous meiosis) where only one out of three or two individuals from the same parent plant showed CNVs not present in the sibling lines, and "inherited" when all two or three individuals of the same line showed the same type of CNV in the same chromosome location (events already fixed by previous meiosis in the parent).

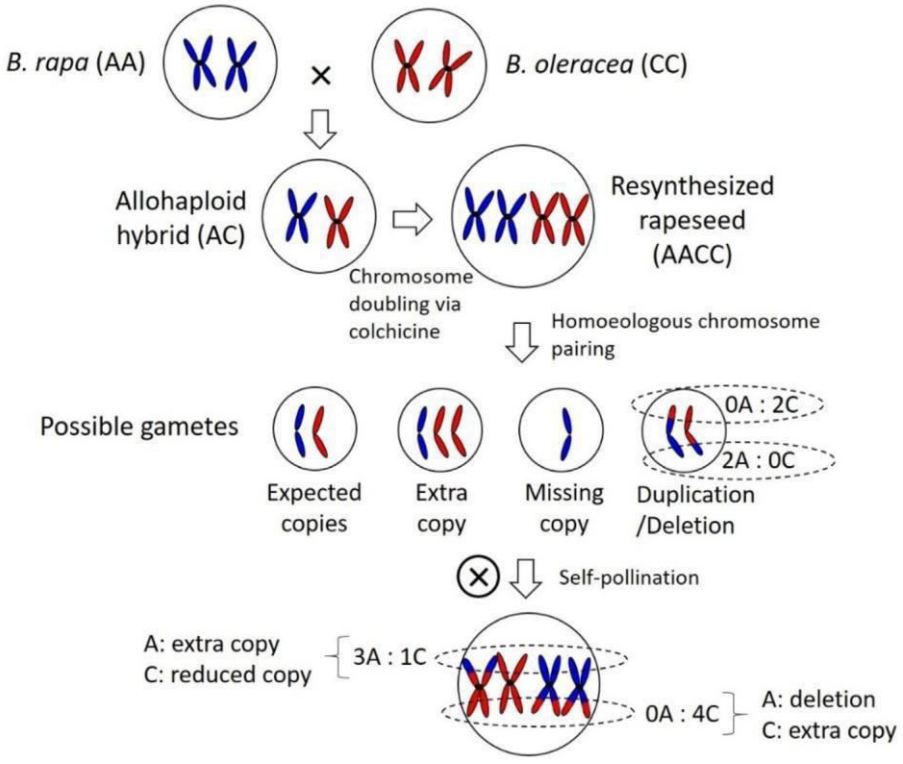

Fig. 2. A simplified crossing scheme and resulting types of copy number variants detected in resynthesized *Brassica napus* lines showing chromosomes or chromosomal segments of a *B. rapa* genotype (red), and a *B. oleracea* genotype (blue).

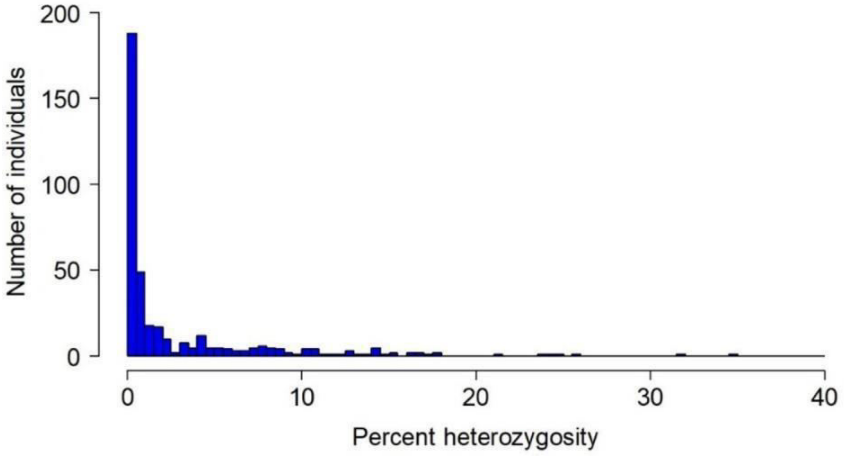

Fig. 3. Percentage heterozygosity in 363 individuals from 116 resynthesized *Brassica napus* lines produced by chromosome doubling of parent F_1 AC hybrids after quality filtering of SNP array genotyping data.

2.6. Statistical analysis

The fertility of resynthesized *B. napus* lines were assessed using the R package version 4.1.0 [57]. We used one-way ANOVA to test for significant differences between resynthesized genotypes in fertility. Tukey's HSD was used to test whether fertility measures such as seeds per ten pods, total number of self-pollinated seeds, and percentage pollen viability as well as total number of CNVs (inherited and novel) were significantly affected by resynthesized genotype. We also used one-way ANOVA to determine whether the number of generations of the lines significantly affected their fertility. We checked for correlations between fertility measures as well as between fertility and CNVs by using the Pearson's correlation coefficient (r) values in R studio. We used Pearson's chi-squared test of independence to determine whether CNV frequency in the pure set had a significant influence on fertility using R studio (library package "MASS"). We manually scored number of both inherited and novel CNVs using logR ratio values of the three individuals per line and produced multiple bar plots in Microsoft Excel 2016.

3. Results

3.1. High rate of contamination was detected in resynthesized B. Napus lines

SNP genotyping was done for all 116 genotypes from which leaf samples were collected. Out of 116 genotypes with SNP genotyping information, 39 genotypes were putatively non-contaminated and homozygous (34%) while 66% were contaminated and/or heterozygous (Figs. 3, S1, S2; Files S3, S4). Out of these, 29 genotypes had at least three putatively pure individuals, seven genotypes contained two putatively pure individuals while three genotypes had only one putatively pure individual each.

3.2. Resynthesized lines show a wide range of genotype-dependent fertility

We grouped resynthesized lines with available genotyping data into putatively contaminated and pure lines, and analysed fertility separately in both groups. Firstly, we analysed 100 contaminated resynthesized lines with available fertility data. Contaminated resynthesized genotypes showed a wide range of fertility as measured by total number of self-pollinated seeds, seeds per ten pods, and percentage pollen viability. Total number of self-pollinated seeds ranged from 0 to 3150 (average 580) (Fig. S3; File S6A). Num- ber of seeds per ten pods also ranged from 0 to 337 with an average of 76.8 per plant. The average percentage pollen viability across resynthesized lines was 88.5%. Putatively contaminated resynthesized *B. napus* genotype significantly affected the total number of self-pollinated seeds, number of seeds per ten pods, and pollen via- bility (ANOVA, $P = 8.68 \times 10^{-11}$, $P = 9.55 \times 10^{-5}$, $P = 2 \times 10^{-16}$, respectively, Tukey's HSD $P < 0.05$). A moderate correlation between number of self-pollinated seeds and number of seeds per ten pods was observed ($r = 0.7$). However, there was no signif- icant association between any of the fertility measures and genetic background (domesticated or wild C genome species) of resynthesized lines (ANOVA, $P > 0.05$). No significant association was found between any of the fertility measures with the direction of the original crossing event (maternal vs paternal species parent).

Secondly, 31 pure resynthesized lines with available fertility data were selected out of 33 pure lines and assessed for fertility. Our result showed a wide range of genotype-dependent fertility as measured by total number of self-pollinated seeds, seeds per ten pods, and percentage pollen viability. Total number of self-pollinated seeds ranged from 0 to 3876 (average 641) (Fig. 4; File S6B). Number of seeds per ten pods ranged from 0 to 292 (average 86). The average percentage pollen viability across pure resynthesized lines was 86.5%. Resynthesized *B. napus* genotypes significantly affected the number of seeds per ten pods, and percentage pollen viability (ANOVA, $P = 7.44 \times 10^{-4}$, $P = 0.000146$, respectively, Tukey's HSD $P < 0.05$) (Tables S2, S3) as well as the total number of self-pollinated seeds (ANOVA, $P = 0.000907$, Tukey's HSD $P > 0.05$). Significant associations were observed between total number of self-pollinated seeds and seeds per ten pods, seeds per ten pods and percentage pollen viability, and between percentage pollen viability and total number of self-pollinated seeds (ANOVA, $P = 1.34 \times 10^{-9}$, $P = 0.00281$, $P = 0.00156$, respectively) with positive correlations (Pearson's correlation coefficient

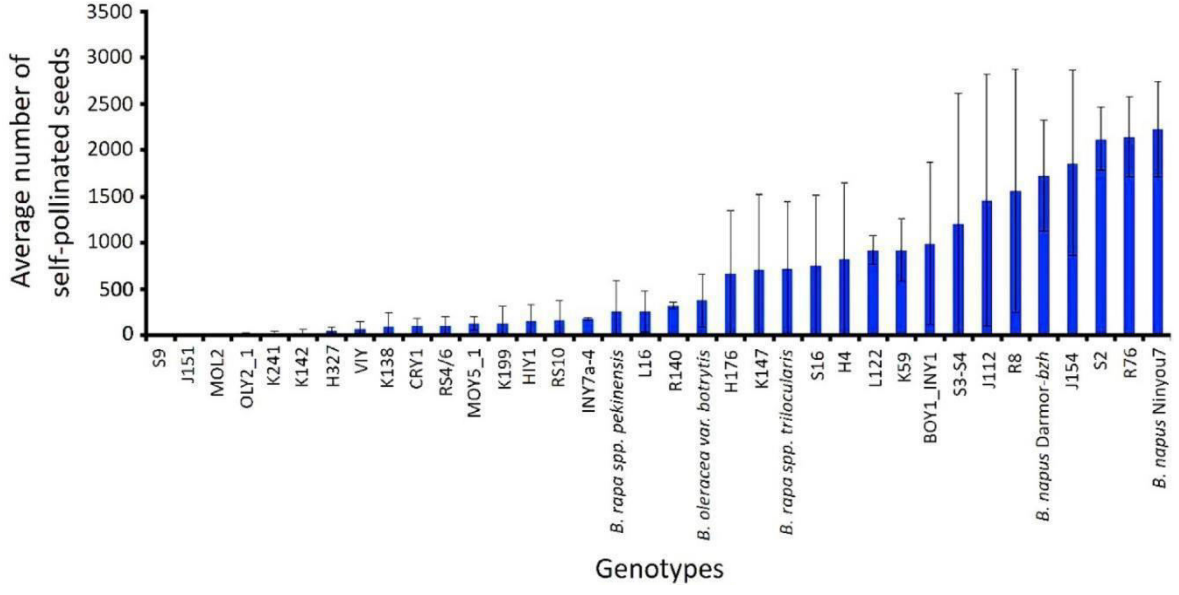

Fig. 4. Fertility of putatively pure resynthesized *Brassica napus* lines measured by the average number of self-pollinated seeds per plant was compared to *B. rapa*, *B. oleracea*, and *B. napus* cultivars.

r = 0.86, 0.53, 0.5, respectively). The highest positive correlation was observed between average number of seeds per ten pods and average self-pollinated seeds (r = 0.86) (Fig. S4). In contrast to the contaminated lines, total number of self-pollinated seeds, and seeds per ten pods were significantly associated with the genetic background (domesticated or wild C genome species) of homozygous and pure resynthesized *B. napus* lines (ANOVA, P = 0.0126, P = 0.0142, respectively) but not percentage pollen via- bility (ANOVA, P = 0.80). Percentage pollen viability, and seeds per ten pods were significantly associated with the direction of the original crossing events between the paternal and maternal parent species (ANOVA, P = 0.0163, P = 0.04, respectively) but not self- pollinated seed set (ANOVA, P = 0.09). Although only a few resyn- thesized *B. napus* genotypes showed comparable fertility to estab- lished *B. napus* cultivars (Fig. 4), higher fertility compared to *B. rapa* and *B. oleracea* cultivars was observed in some resynthesized genotypes.

3.3. Fertility of resynthesized B. napus lines is significantly affected by generation

Out of 33 pure lines, 31 homozygous, pure resynthesized lines with fertility data were selected and assessed in order to determine whether fertility of the lines was affected by the number of gener- ations in which the lines have been produced. We found that although there was significant difference in total seed set across generations, total seed set did not significantly improve across gen- erations (Fig. 5A) (ANOVA, P = 0.01, Tukey's HSD P < 0.05). Percent- age pollen viability and seeds per ten pods showed improved fertility across generations (Fig. 5B, C): although there was no sig- nificant difference between the first two generations, later genera- tions showed improved seed fertility as measured by seeds per ten pods (Fig. 5C) (ANOVA, P = 0.0008, P = 0.001, respectively, Tukey's HSD, P < 0.05).

3.4. High frequency of copy number variants detected in resynthesized B. napus lines

We detected copy number variants (deletion, reduced copy, and higher copy) across the S_1 and older generations of the 33 puta- tively pure resynthesized *B. napus* lines with at least two individu- als and fertility data. Resynthesized *B. napus* lines analysed showed a high frequency of CNV in the A and C genomes (Fig. S5). The fre- quency of CNV varied between resynthesized lines. Resynthesized *B. napus* genotype significantly affected the total number of CNVs (ANOVA, $P = 1 \times 10^{-16}$). Higher numbers of CNVs were significantly associated with reduced fertility as measured by total number of self-pollinated seeds, seeds per ten pods, and percentage pollen viability (ANOVA, $P = 1 \times 10^{-8}$, $P = 1 \times 10^{-7}$, $P < 1 \times 10^{-5}$ and Pear- son's chi-squared test, P < 0.01, P = 0.01, P < 0.01, respectively) (Tables S4–S6). CNV frequency showed a moderate correlation with the average number of self-pollinated seeds (r = −0.61) (Fig. S6).

We also observed that novel and inherited copy number vari- ants both occurred genome-wide across the A and the C genomes, except on chromosome A08 where only novel CNVs were found. The frequency of novel and inherited CNVs was higher for homoe- ologous chromosomes A01/C01, A02/C02, A03/C03, and A09/C09 (Fig. S5). We also observed CNVs affecting entire or nearly whole chromosomes (File S5).

3.5. Putatively stable genotypes observed in resynthesized B. napus

We assessed 33 pure resynthesized lines in order to score novel and inherited CNVs using logR ratios. We observed that individuals

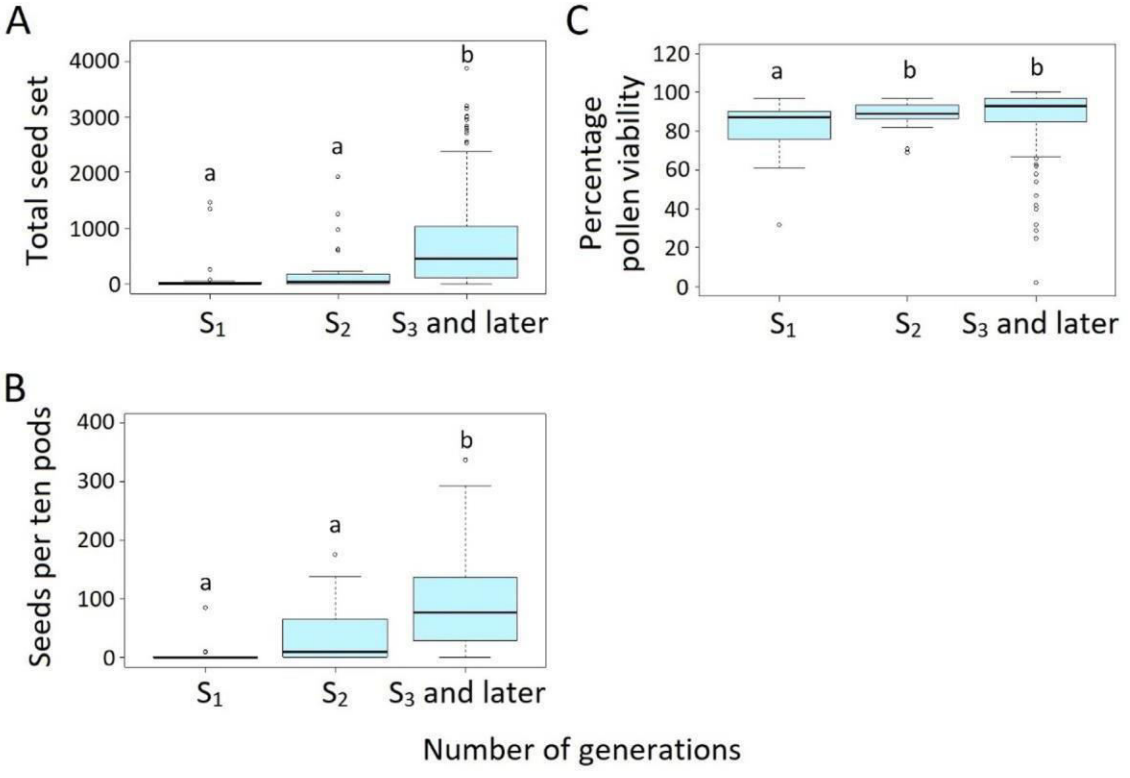

Fig. 5. Association of fertility to number of generations of resynthesized lines produced was measured by: (A) Total seed set (B) Seeds per ten pods (C) Percentage pollen viability. (ANOVA, P = 0.01, P < 0.01, P > 0.01, respectively, Tukey's HSD P < 0.01).

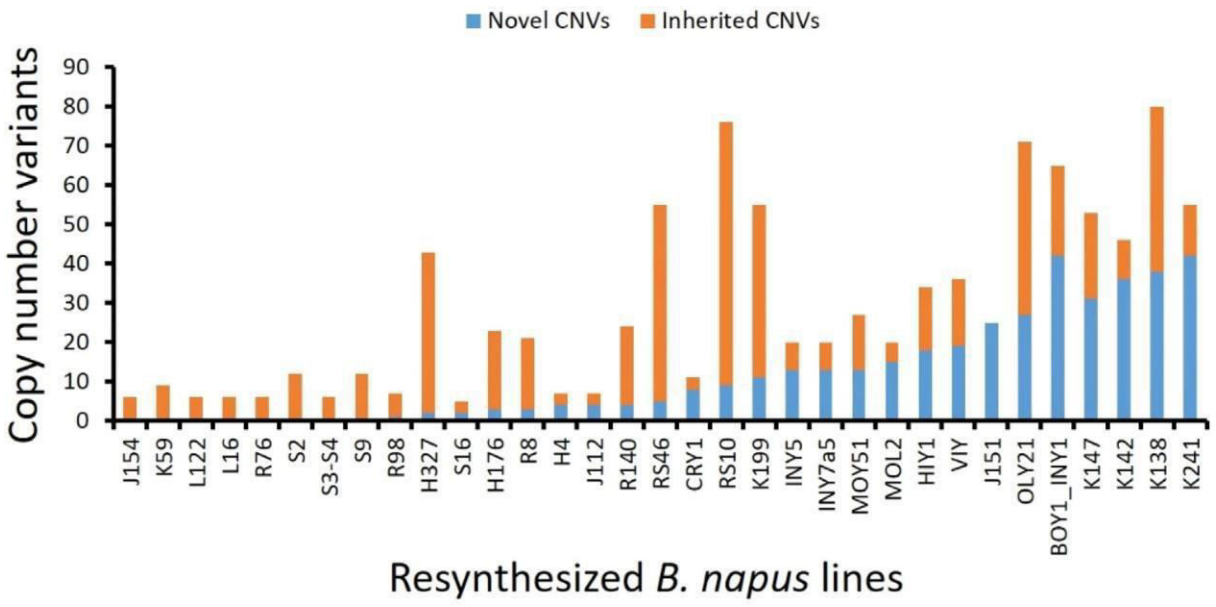

Fig. 6. Number of copy number variants showing both novel (blue), and inherited (red) copy number variants in 33 resynthesized *Brassica napus* lines.

of the same resynthesized line analyzed showed both novel and/or inherited CNVs (Fig. 6; File S7).

4. Discussion

In this study, we analyzed fertility by scoring seeds per ten pods, percentage pollen viability, and total number of self- pollinated seed-set as well as genome stability as measured by both inherited and novel copy number variants in resynthesized *B. napus* lines. Results showed the presence of both novel and inherited copy number variants as well as a wide-range of geno- type dependent fertility across generations in resynthesized *B. napus* lines. We also found that fertility was significantly affected by number of generations. Most genotypes were contaminated by outcrossing to unknown parents; this may be a common fate for poorly self-fertile resynthesized lines. However, some lines were also putatively stable, and may be maintained indefinitely as breeding or research germplasm without accumulation of non-homologous chromosome rearrangements.

Although *B. napus* is a self-compatible species, unlike its diploid *B. rapa* and *B. oleracea* progenitors [58,59], a few studies have reported incidences of heterozygosity as a result of outcrossing in the field [60] or via seed contamination [61,62]. In this study, we observed 72% contamination as estimated by the rate of heterozygosity (AB) across lines in our SNP data, possibly as a result of outcrossing with established *B. napus* on the field where the lines were initially grown [4,24]. Newly resynthesized lines are often partially self-incompatible, which can explain the low fertility after self-pollination, although our resynthesized lines generally had high percentage pollen viability, with a few exceptions (Fig. 5). When multiplying partially self-incompatible lines there is a high risk of uncontrolled outcrossing. Cresswell et al. [63] also observed a high rate of over 80% out-crossing following bee pollination in a self-compatible *B. napus* variety. Bayer et al. [64] analyzed 92 double-haploid individuals from a Tapidor x Ningyou 7 mapping population, and detected an unex- pectedly high frequency of heterozygous alleles in 25 individuals, mostly attributed to outcrossing between lines during population development. Previous studies have analyzed 20 synthetics out of the resynthesized lines used in our study for genome-wide studies to detect genome-wide SNPs as well as study chromosome rearrangement and homoeologous exchanges between the A and C subgenomes [65,66]. However, these studies did not screen for homozygosity in the synthetic lines used. Our results using the same resynthesized lines showed that 65% of the lines used by [65,66] were probably contaminated. Single outcrossing events in established lines do not necessarily pose a huge problem for breed- ing provided that the exotic allelic contribution is still retained in the recombined progeny, but widespread contamination and unknown parentage hinders efforts to preserve germplasm as well as undertake genetic analysis.

In this study, resynthesized *B. napus* lines showed a wide-range of genotype-dependent fertility as well as higher fertility than *B. rapa* and *B. oleracea* cultivars. Similarly, a few studies in synthetic allotetraploid *Brassica* populations have also observed genotype-dependent fertility [13,67]. Malek et al. [29] analyzed 40 synthetic *B. napus* plants produced from a single genotype of *B. rapa* ssp. *trioc- ularis* and *B. oleracea* var. *alboglabra* using number of seeds per silique, 1000-seed weight and seed yield per plant as fertility measures and found that fertility was higher than that of *B. rapa* and *B. oleracea* parents, similar to our results. In contrast, Karim et al. [12] analysed 17 resynthesized F_1 plants, self-pollinated to the F_3 generation derived from five different cross combinations of *B. rapa* and *B. oleracea* and observed lower number of seeds per siliques compared to both established *B. napus* cultivars and relative to parental *B. rapa* and *B. oleracea* cultivars, although increased number of pods per plant was observed [12]. Girke et al. [13] produced and analysed hybrids produced from crosses between 44 resynthe- sized *B. napus* lines with diverse parental origins and two male- sterile winter oilseed rape tester lines for heterosis and found that a number of hybrids had higher yields than the mean yield of check cultivars. We also found that some of our lines were more fertile than *B. rapa* and *B. oleracea* cultivars, although only a few resynthe- sized lines had higher or comparable fertility to established *B. napus* cultivars. Hypothetically, the resynthesized lines may have acquired this increased fertility over generations of self- pollination and under preselection for fertility; poorly-fertile lines may have died in the field or glasshouse without setting seed.

We found that the fertility of our resynthesized *B. napus* (as measured by seeds per ten pods and percentage pollen viability) was significantly higher in later generation lines (S_3+) than in the S_1 and S_2 genotypes. Several studies on synthetic *Brassica* allopolyploids have shown that fertility and meiotic stability are significantly affected over subsequent generations [27,48–51,68,69]. Rousseau-Gueutin et al. [27] assessed fertility based on the number of seeds per 100 flowers in in both open-pollinated and self-fertilized resynthesized *B. napus* lines and observed that fertility was significantly reduced over subsequent generations (S_1 to S_3) except in self-fertilized plants. By contrast, Xiong et al. [48] analysed later generation resynthesized lines (S_{10} to S_{11}) for fertility and observed a decrease in fertility with successive generations using seed set and pollen viability as fertility measures. Other studies in synthetic *Brassica* hybrids also observed increased fertility similar to our study: Katche et al. [49] observed increased seed set and pollen viability across the F_1 to S_4 in BBAC (*B. juncea* × *B. carinata*) interspecific hybrids produced over six generations of self-pollination under selection for high fertility. Gaebelein et al. [50] analyzed mapping populations of synthetic allohexaploids (*B. napus* × *B. carinata*) × *B. juncea* and observed increased seed production from H_1 to $H_{3/4}$ in two of four cross combination with selection. Similarly, a previous study in *Brassica* allohexaploid hybrids (*B. carinata* × *B. rapa*) also reported increased pollen fertility and seed number per pod from H_2 to H_4 generations [51]. However, reports of increased fertility were always linked to initially heterozygous starting material, where selection for allelic variants linked to fertility could take place. For initially homozygous lines, only copy number variants can act as a selective substrate, and these are mostly expected to be deleterious [56]. Hence, from our study, it seems most likely that early-generation resynthesized lines with poor fertility are simply less likely to survive to later generations.

We detected both novel and/or inherited copy number variants in homozygous resynthesized *B. napus* lines and found significant association between copy number variation and all fertility measures such as seeds per ten pods, total number of self-pollinated seeds and percentage pollen viability. Copy number variation refers to the presence of DNA sequences in copies usually larger than 1 kb which varies in number among individuals of the same species [70]. Copy number variation has been shown in many polyploids to influence the phenotypic variants of major agronomic traits such as flowering time [71,72], plant height [73], stress tolerance [74], and resistance [75]. Completely homozygous lines such as double haploids and highly inbred lines are suitable plant resources for studying CNVs and their impact on plant phenotypes [76]. Gaebelein et al. [50] analysed synthetic *Brassica* allohexaploid lines and found out that the loss or retention of chromosomes present only in a single copy had the greatest influence on the plant fertility. Schiessl et al. [77] analysed a diversity set of 280 genetically diverse *B. napus* inbred lines using sequence capture and detected the presence of CNVs in 35 flowering time regulator genes. Zhang et al. [78] analysed a double haploid population of established *B. napus* and found that a 24,482-bp deletion on chromosome C09 was probably responsible for a 1000-seed weight trait qSW.C9. Zhang et al. [78] suggested that the regulation of qSW.C9 is likely attributed to either the presence of copy number variants or presence/absence variants. Many phenotypic traits have also been associated with the presence of CNVs in domestic animals [79]. In this study, we showed that CNVs are abundant in resynthesized *B. napus* lines, and associated higher number of CNVs with reduced fertility.

We detected higher frequencies of novel and inherited copy number variation in the chromosomes with larger stretches of homoeology between the A and C subgenomes, as expected from previous studies on resynthesized *B. napus* [48,80]. Here, we observed higher frequencies of novel and inherited CNVs in homoeologous chromosomes A01-C01, A02-C02, A03-C03, and A09-C09 compared to other chromosomes. No inherited CNVs were observed on chromosome A08. Samans et al. [66] also found higher frequencies of homoeologous exchanges between A1-C1, A2-C2, A3-C3, A9-C8, and A9-C9 in synthetic *Brassica napus*. We assume based on our results that these homoeologous chromo- somes probably accumulate more CNVs as a result of more fre- quent A-C chromosome pairings. This is corroborated by observations of more CNVs in the C subgenome than in the A sub- genome, similar to [66] where more rearrangements were also found in the C subgenome than in the A subgenome by analysing a subset of the same synthetic *B. napus* lines used in our study. Bias towards loss of the C genome is commonly observed in *Brassica* synthetics [49,50] and may be linked to selective advantage to retain the A genome [50,81].

We observed no novel CNVs in a few of our synthetic *B. napus* lines. Interestingly, all of these lines which showed no novel CNVs belong to the domesticated groups of resynthesized lines already grown for more than two generations. Studies in *Brassica* allopolyploids have shown that meiotic stability was attained in some synthetic *Brassica* hybrids after a few generations of crossings or self-pollination [49,50,82]. In fact, a few studies have shown that it is possible for synthetics and newly formed polyploids to be immediately stable following polyploidization [83–85]. Gupta et al. [83] analysed *Brassica* hexaploids from a cross between *B. carinata* and *B. rapa* and found a meiotically stable genotype. With regards to the present study, the detection of no novel CNVs in a few lines possibly indicates that our resynthesized lines inherited allelic variants from the *B. rapa* and *B. oleracea* parents that conferred immediate meiotic stability [42].

5. Conclusions

Resynthesized *B. napus* lines produced from domesticated *B. rapa* and *B. oleracea* parental lines, as well as wild C genome spe- cies, are highly useful resources for increasing the genetic diversity of established rapeseed, and for the introgression of useful adap- tive traits into the current rapeseed pool. In this study, we charac- terized and analysed a large set of resynthesized *B. napus* lines for homozygosity, fertility and genome stability. Most lines showed evidence of unintended outcrossing, highlighting that open pollination is extremely frequent even under controlled conditions in poorly fertile resynthesized lines. Our results showed that both fertility and genome stability are significantly associated with genotype, and suggest that some resynthesized lines may in fact be stable and fertile, and can hence be maintained for many genera- tions with limited non-homologous recombination as resources for research and breeding.

CRediT authorship contribution statement

Elizabeth Ihien Katche: Methodology, Investigation, Writing- original draft. Antje Schierholt: Resources, Writing- review and editing. Heiko C. Becker: Resources, Writing- review and editing. Jacqueline Batley: Methodology, Writing- review and editing. Annaliese Mason: Conceptualization, Funding acquisition, Supervision, Writing- review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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4.0 Genetic factors inherited from both diploid parents interact to affect genome stability and fertility in resynthesized allotetraploid *Brassica napus*

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4.1 Publication Outline

The following study tested the hypothesis that allopolyploids may have inherited specific allelic variation from their diploid progenitors which conferred meiotic stability by analyzing 41 resynthesized *B. napus* lines produced by crosses between eight *B. rapa* and eight *B. oleracea* for copy number variation and fertility. We then resequenced eight *B. rapa* and five *B. oleracea* parent lines and assessed 19 resynthesized lines for allelic variation in a list of meiosis gene homologs. Self-pollinated seed set and genome stability (as measured by copy number variants) were significantly associated with the interaction between both *B. rapa* and *B. oleracea* parental genotypes. Thirteen putative meiosis gene candidates were detected which showed significant association with CNV frequency and the presence of putatively harmful mutations in meiosis gene haplotypes.

4.2 Publication

Authors contribution

Resynthesized material used in this study was produced at the university of Goettingen (AS and HB) AM conceptualised the experiment with help from AS. EKI grew the plants, collected and analysed the fertility and SNP genotyping data with generation of the latter carried out by JB, and drafted the paper. SVS, FH and ZL analysed sequencing data, and SVS and FH provided final nalysis and results related to meiosis gene variation. AM, AS, HB, ZL, and SVS contributed to critical revisions of the manuscript.

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Investigation

Genetic factors inherited from both diploid parents interact to affect genome stability and fertility in resynthesized allotetraploid *Brassica napus*

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Abstract

Established allopolyploids are known to be genomically stable and fertile. However, in contrast, most newly resynthesized all opolyploids are infertile and meiotically unstable. Identifying the genetic factors responsible for genome stability in newly formed allopolyploid is key to understanding how 2 genomes come together to form a species. One hypothesis is that established allopolyploids may have inherited specific alleles from their diploid progenitors which conferred meiotic stability. Resynthesized *Brassica napus* lines are often unstable and infertile, unlike *B. napus* cultivars. We tested this hypothesis by characterizing 41 resynthesized *B. napus* lines produced by crosses between 8 *Brassica rapa* and 8 *Brassica oleracea* lines for copy number variation resulting from nonhomologous recombination events and fertility. We resequenced 8 *B. rapa* and 5 *B. oleracea* parent accessions and analyzed 19 resynthesized lines for allelic variation in a list of meiosis gene homologs. SNP genotyping was performed using the Illumina Infinium *Brassica* 60K array for 3 individuals per line. Self-pollinated seed set and genome stability (number of copy number variants) were significantly affected by the interaction between both *B. rapa* and *B. oleracea* parental genotypes. We identified 13 putative meiosis gene candidates which were significantly associated with frequency of copy number variants and which contained putatively harmful mutations in meiosis gene haplotypes for further investigation. Our results support the hypothesis that allelic variants inherited from parental genotypes affect genome stability and fertility in resynthesized rapeseed.

Keywords: copy number variation, fertility, genome stability, meiosis, resynthesized *Brassica napus*, single nucleotide polymorphism

Introduction

Polyploidy is the heritable condition of possessing more than 2 sets of chromosomes (Comai 2005). The extra set/s of chromosomes may originate from the same individual or from within the same species, which is referred to as autopolyploidy, or from hybridization between 2 different species, known as allopolyploidy (Otto 2007). Polyploidy confers a number of evolutionary advantages (Soltis and Soltis 2000), including increased potential for heterosis due to the contribution of additional gene copies to a trait, and genetic redundancy which allows additional gene copies to take on new functions without loss of established and required functions in the organism (reviewed by Comai 2005). Polyploids do however face significant challenges to establishment, including initial self-propagation, and reproductive isolation from and competition with parental progenitor species (reviewed by Mable 2013 and Shimizu 2022). One of the most significant barriers to polyploid establishment is thought to be regulation of meiosis (Pelé *et al.* 2018). In newly formed polyploids, cell machinery must adapt to the presence of extra chromosomes: in autopolyploids via enforcement of a single crossover per chromosome pair per meiosis and in allopolyploids via strict segregation of homologous chromosomes belonging to each of the progenitor genomes (prevention of nonhomologous chromosome pairing) (reviewed by Bomblies 2023).

How the process of meiotic adaptation to polyploidy occurs is still relatively unknown across most taxa, although insights into the genetic mechanisms involved have now been gained in several species (reviewed by Bomblies 2023). In bread wheat (*Triticum aestivum*), prevention of homoeologous crossovers is known to be regulated by the *Ph1* locus (Griffiths *et al.* 2006), which contains a duplicated and diverged copy of meiosis gene *ZIP4* (*TaZIP4-B2*), a gene which is essential for homologous crossover formation as well as synapsis in wheat (Martín *et al.* 2021). In autopolyploid *Arabidopsis arenosa*, 8 meiosis genes were initially identified as under selective sweeps related to adaptation to polyploidy (Yant *et al.* 2013); of these, polyploid-adapted alleles of meiotic chromosome axis formation genes *ASY1* and *ASY3* (Morgan *et al.* 2020) as well as interacting chromatin condensation and axis recruitment gene *REC8* (Morgan *et al.* 2022) have so far been shown to act in stabilizing polyploid meiosis.

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The *Brassica* genus is 1 of 51 genera in the tribe Brassiceae belonging to the crucifer family (Brassicaceae) and is the most economically important genus within this tribe (Rakow 2004). It is an interesting model for allopolyploid formation in agricultural crops as 6 agriculturally significant species share a genomic interrelationship (U 1935). *Brassica napus* (genome $A_nA_nC_nC_n$) was spontaneously formed by recent allopolyploidy between ancestors of *Brassica oleracea* (Mediterranean cabbage, genome C_oC_o) and *Brassica rapa* (Asian cabbage or turnip, genome A_rA_r) in the last 7,500 years and is thought to be polyphyletic in origin (Allender and King 2010; Chalhoub *et al.* 2014). Therefore, the *Brassica* genus and most especially *B. napus* are increasingly receiving attention as a model for regulation of meiosis in a young polyploid crop species (Mason and Snowdon 2016).

To date, some progress has been made in identifying potential causes for the stabilization of meiosis in allopolyploid *B. napus*, which is thought to be under quantitative genetic control, similar to Brassicaceae relative *Arabidopsis* species (Liu *et al.* 2006; Higgins *et al.* 2021; Morgan *et al.* 2022). Although no molecular characterization of gene candidates has been carried out as in *Arabidopsis* and bread wheat, quantitative trait loci mapping approaches have revealed at least 1 major-effect locus (contributing 32–58% to homoeologous recombination frequency), for which a differentially expressed meiosis gene candidate *RPA1C* was identified (Higgins *et al.* 2021). Recent work with mutation (knockout) lines has also suggested that many different pathways to meiotic stabilization may be possible within *Brassica* allopolyploids (Gonzalo *et al.* 2019; Gonzalo 2022). As well, retention of meiosis gene copies following earlier polyploidization events in the *Brassica* lineage (Lloyd *et al.* 2014) and the presence of preexisting meiotic gene variants in diploid species which may contribute to enhanced stabilization of meiosis in the polyploid have been suggested as possible mechanisms for meiotic stabilization (Cifuentes *et al.* 2010).

Synthetic polyploids can be produced through genome doubling of diploid plants or hybrids via methods such as chemical treatment with colchicine (Spoelhof *et al.* 2017). However, most synthetic polyploids remain largely unstable in terms of meiosis and genome inheritance (reviewed by Pelé *et al.* 2018). Meiotic aberrations are common in newly formed autopolyploid and allopolyploid plants, which negatively affects their fertility and early demograph- ic success (Ramsey and Schemske 2002; Gaeta and Pires 2010). Synthetic allopolyploids lack the phenotypic and genomic stability of established allopolyploids (Soltis and Soltis 1995; Pikaard 1999; Comai *et al.* 2000). Abnormal phenotypes and frequent failure of pollen and embryo development (Schranz and Osborn 2000; Comai *et al.* 2003) as well as widespread changes in gene expression have been observed in other synthetic allopolyploids (Kashkush *et al.* 2002). Although not all newly resynthesized allopolyploids are genomically unstable (Comai *et al.* 2000; Wang *et al.* 2006; Novikova *et al.* 2017; Chen *et al.* 2020), most are, including synthetic *Brassica* allotetraploids (Song *et al.* 1995), and this has been attributed to abnormal meiosis (Szadkowski *et al.* 2010). This meiotic instability involves homoeologous pairing or interactions between the closely related A and C genome chromosomes during meiosis (Comai *et al.* 2000; Nicolas *et al.* 2007, 2012; Leflon *et al.* 2010). Although several studies have produced and investigated synthetic *Brassica* (e.g. Abel *et al.* 2005; Szadkowski *et al.* 2010; Mason *et al.* 2010; Girke *et al.* 2012; Jesske *et al.* 2013; Karim *et al.* 2014), almost all synthetic *Brassica* lines investigated so far appear to be meiotically unstable (Chen *et al.* 2011; Gaebelein and Mason 2018).

The question then is why the established *B. napus* species is stable and the resynthesized lines are unstable. One hypothesis is that *B. napus* may have gained genetic control via the inheritance of specific alleles from its diploid progenitor species, while a competing hypothesis suggests that mutations in the newly formed *B. napus* allopolyploid were selected on to restore meiotic stability (reviewed by Cifuentes *et al.* 2010). An increase in mutations as a result of interspecific hybridization (also known as "genomic shock") has been established in several species (reviewed by Jackson and Chen 2010 and Soltis *et al.* 2016), famously maize (McClintock 1984), and nonhomologous translocations that have been observed in synthetic *B. napus* (Gaeta *et al.* 2007; Xiong *et al.* 2011; Samans *et al.* 2017) may also comprise a mechanism for novel mutations to restore meiotic function. The lack of observation of consistent translocations or other fixed genomic rearrangements in established *B. napus* relative to progenitor species *B. rapa* and *B. oleracea* (Chalhoub *et al.* 2014; Samans *et al.* 2017) fails to provide support for this hypothesis, although other types of as-yet-undetected mutations (e.g. transposable element-induced) in the newly formed resynthesized lines may also be responsible (Zou *et al.* 2011; Fu *et al.* 2016). These 2 hypotheses are also not exclusive, and recent reports in *Arabidopsis* have also suggested that evolution of meiotic stability may be a more gradual process, with polygenic selection for allelic variants, novel mutations, or regulations of gene expression that improve regularity of chromosome recombination and segregation and hence genome stability and fertility (Burns *et al.* 2021; Morgan *et al.* 2021).

A few studies have been conducted to explain genome instability in resynthesized *B. napus* allotetraploids (Gaeta *et al.* 2007; Szadkowski *et al.* 2010; Xiong *et al.* 2011), and recently several quantitative trait loci were identified to be present in natural *B. napus* that confer reduced homoeologous recombination rates (Higgins *et al.* 2021). However, no study to date has investigated multiple genotypes of resynthesized *B. napus* in order to test the idea that allelic variation inherited from the progenitor species conferred meiotic stability to natural *B. napus*. In this study, we aimed to test the hypothesis that allelic variants inherited from diploid progenitor species *B. rapa* and *B. oleracea* affect the frequency of homoeologous recombination in resynthesized *B. napus*, and hence, that inherited allelic variation may have conferred genomic stability to resynthesized *B. napus* lines.

Materials and methods

Description of plant material

The materials used in this study comprise resynthesized *B. napus* seeds derived from crosses between homozygous *B. rapa* and *B. oleracea* parents as described in Abel *et al.* (2005), where these are referred to as "spring-type domesticated lines." The parental genotypes are either doubled haploid or highly inbred lines. C genome genotypes are either cauliflower (*B. oleracea* var. *botrytis*) or Chinese kale (*B. oleracea* var. *alboglabra*), and A genome genotypes are yellow sarson (*B. rapa* ssp. *trilocularis*), oilseed turnip (*B. rapa* ssp. *oleifera*, listed as *B. rapa* var. *rapa* in Abel *et al.* 2005), and Chinese cabbage (*B. rapa* ssp. *pekinensis*) (Supplementary File 1). Abel *et al.* (2005) produced seeds from 336 cross combinations between 21 *B. rapa* (maternal parent) and 16 *B. oleracea* lines including a core set of 64 cross combinations between 8 *B. rapa* and 8 *B. oleracea* lines. *B. rapa* was always the maternal parent in the crosses. Seeds from 41 resynthesized *B. napus* genotypes produced from crosses between 8 *B. rapa* (A4, A6, A7, A8, A9, A13, A16, and A19) and 8 *B. oleracea* parent genotypes (C34, C36, C37, C38, C42, C46, C47, and C49) via embryo rescue, chromosome doubling and self-pollination (S_1 generation),and their *B. rapa* and *B. oleracea* parent genotypes were used in this study. Established *Brassica* cultivars were used as controls for fertility in our experiment: winter-type

B. napus 'Darmor', spring-type *B. napus* 'Argyle', and semiwinter-type *B. napus* 'Ningyou7', as well as *B. rapa* var. *oleifera* (unknown accession) and *B. oleracea* var. *botrytis* 'NGB 1810.2'.

The resynthesized *B. napus* lines are represented by codes in the form "A1C1," where "A1" is the *B. rapa* parent genotype and "C1" is the *B. oleracea* parent genotype. In the present study, 3 seeds from each of 41 resynthesized genotypes and cultivars of established *B. napus*, *B. rapa*, and *B. oleracea* used as controls were sown in quick pots and seedlings transferred to small pots without vernalization between September and November 2017 under glasshouse conditions at Justus Liebig University (JLU). Eight *B. rapa* parent genotypes (A4, A6, A7, A8, A9, A13, A16, and A19) as well as 6 *B. oleracea* lines (C34, C36, C37, C38, C46, and C47) used to produce the resynthesized *B. napus* lines were likewise sown on 2019 September 12 under the same glasshouse conditions at JLU as the resynthesized lines. All lines except C38 successfully germinated and produced plants. Three plant replicates from each genotype were then isolated in bags at flowering to ensure self-pollination.

Assessment of purity in resynthesized *B. napus* lines

The purity of 41 resynthesized *B. napus* genotypes with SNP genotyping information was assessed. Eight parent *B. rapa*, 5 double haploid (DH) lines (A6, A7, A8, A9, and A13) and 3 inbred lines (A4, A16, and A19), as well as 8 *B. oleracea* genotypes, 5 DH lines (C34, C36, C37, C38, and C42) and 3 inbred lines (C46, C47, and C49), were used to produce the resynthesized lines (Abel *et al* 2005). Resynthesized lines produced by DH parental crosses between *B. rapa* and *B. oleracea* (AA × CC) are expected to be completely homozygous. Therefore, individuals of the same progeny sets having the same maternal *B. rapa* (AA) or paternal *B. oleracea* (CC) parents are expected to be nonsegregating. We assessed pur- ity using 2 criteria: (1) the absence of segregation pattern among progeny sets and (2) the absence of continuous blocks of heterozygous (AB) calls across the A and the C genomes in all individuals.

Fertility assessment in resynthesized *B. napus* lines

Three parameters were scored to describe fertility in resynthesized lines: relative pollen viability (as estimated by stainability), total number of seeds produced per plant, and number of seeds produced per 10 pods. Pollen viability was assessed for 2 freshly opened flowers per plant, and pollen grains stained with 1–2% acetocarmine solution (Leflon *et al.* 2006). At least 600 pollen grains per plant were counted and pollen viability was assessed using a light microscope (Leica DMR, Leica Microsystems), assuming darkly stained (red) pollen grains were viable and weakly stained or shrivelled pollen grains were nonviable. The total number of self-pollinated seeds produced per plant and the number of seeds produced per 10 pods were counted for each plant after harvesting. Individual plants were bagged after initiation of flowering using microperforated plastic bags to encourage self-pollination. The measure of total number of seeds per plant was collected as a very rough approximation of yield, while seeds per pod were assumed to relate better to meiotic process and % development of viable embryos.

DNA extraction and genotyping using the Illumina Infinium *Brassica* 60K SNP array

Young leaf samples were collected in 2 mL microcentrifuge tubes at the 4–6 leaf stage of plant development. DNA was extracted for 41 resynthesized lines (123 plants) using the BioSprint 96 plant work station (Qiagen, Hilden, Germany) according to the manufacturer's instructions (http://qiagen.com/). SNP genotyping was carried out using the high-throughput Illumina Infinium 60K *Brassica* SNP array for the resynthesized lines. Hybridization protocols were performed according to the manufacturer's instructions for all samples.

SNP data were analyzed, visualized, and exported into text files using Genome Studio v2.0.4 software (Illumina Inc., San Diego, CA, USA). All 52,149 SNPs were exported for the A and C genomes after application of the recommended "brassica60K" cluster file (Clarke *et al.* 2016). Top BLAST alignment hits for the SNP probes against the A and C genomes of the reference genome sequence of Darmor-*bzh* version 8.1 (Bayer *et al.* 2017) were used for genome position information. Hits to unplaced contigs were removed from further analyses. Data from samples of each *Brassica* species sourced from Mason *et al.* (2015) were used as controls for filtering SNPs. SNPs which mapped to the A genome but which amplified in *Brassica carinata* (BBCC) and *B. oleracea* (CC) genotype controls as well as SNPs which mapped to the C genome but amplified in *Brassica juncea* (AABB) and *B. rapa* (AA) genotype controls in >50% of the controls were filtered out. SNP markers with >50% heterozygous AB calls in all 5 *B. napus* homozygous control cultivars (Boomer, Monty_028DH, Surpass400_024DH, Trilogy, and Westar_10DH) as well as SNPs which showed >99% missing calls (NC) across all lines were removed. Further filtering steps included the removal of SNPs with ≥80% AB calls across individuals. After filtering, 21,938 SNPs were retained: 8,369 SNPs in the A genome and 13,569 SNPs in the C genome (Supplementary File 2). Genotype calls were then converted to homozygous/heterozygous calls (0 and 2 for homozygous and 1 for heterozygous) and incidence of missing calls represented by NA. Since the resynthesized lines were produced by chromosome doubling of parent F_1 AC hybrids using colchicine, the allotetraploid hybrids are expected to be homozygous. After quality filtering of SNPs, we used the filtered SNPs to plot dendrograms separately for both the A and C genome parents (Supplementary Figs. 1 and 2).

Detection of copy number variation in resynthesized *B. napus* lines

A copy number pipeline was developed in R (Schiessl *et al.*, unpublished). The pipeline uses the log *R* ratios (Supplementary Files 3 and 4) to make plots for every individual line based on estimated cutoff values to score copy number variants. Log *R* ratios estimate relative fluorescence intensity for each SNP marker and are an output metric of Illumina GenomeStudio, the program used to call SNPs from raw data. For every SNP, we screened a diverse population representative for the diversity among natural *B. napus* (ASSYST) to get the expected log *R* ratio (Bus *et al.* 2012; Körber *et al.* 2012) distribution for this specific SNP. We then use quantiles to determine if the log *R* ratio of the SNP in our test population is unexpectedly low or high. The quantiles used for "deletion" were 10, for "missing copy" were 25, and for "extra copy" were 75: if SNP x in line y had a log *R* ratio value lower than the expected 10th quantile, SNP x was marked as a threshold SNP in line y. Windows with more than 5 threshold SNPs were kept and merged in case of physical overlap. The merged regions were reevaluated for the threshold SNP content, and regions with >50% threshold SNPs were retained. Regions with the same copy number variant (CNV) direction (extra copy/copies, or missing regions/deleted regions) that were very close together (<5 Mb) were merged (declared as deletion in the case that a deletion and a missing copy region were merged), and regions <2 Mb were also filtered out, so as not to overestimate CNV numbers based on noise in the data, especially since we only expect a limited number of nonhomologous recombination events per chromosome and hence smaller CNVs are less likely in this population (2 close-together

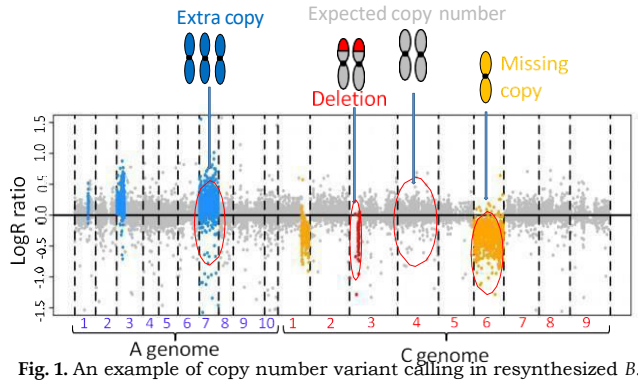

Fig. 1. An example of copy number variant calling in resynthesized *B. napus* lines showing regions of higher copy number (blue: small regions on chromosomes A01 and A03 and all of chromosome A07), no copies/deletion (red: start of chromosome C03), expected copy (gray: majority of markers, e.g. chromosome C4), and single/reduced copy (orange: latter part of chromosome C1 and all of chromosome C6). Discrimination between 1 or 2 additional copies (3 or 4 copies total) was not possible using this method; these are hence referred to as "higher copy number" regions.

nonhomologous recombination events are required for a small, nontelomeric CNV). Therefore, using the abovementioned criteria, we assessed CNVs in the resynthesized *B. napus* lines as deletion, missing/reduced copy, and extra/higher copy, with 2 copies as the expected copy number (Fig. 1). We also screened the log *R* ratio data of the resynthesized *B. napus* lines to check whether the same CNVs (inherited) are present in all progeny set derived from the *B. rapa* or *B. oleracea* parents.

DNA extraction, sequencing, and sequence analysis of parental lines

DNA was extracted from young leaf samples of 8 *B. rapa* genotypes (A4, A6, A7, A8, A9, A13, A16, and A19) as well as 5 *B. oleracea* genotypes (C34, C36, C37, C46, and C47) using the Doyle and Doyle (1987) DNA extraction protocol. Illumina paired-end sequencing was performed at Novogene Company Limited, United Kingdom, on an Illumina HiSeq machine to produce reads of 150 bp length. Quality control was performed using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and no further processing was found to be necessary. The parental reference genomes *B. rapa* cv. Chiifu version 3.0 (Zhang *et al.* 2018) and *B. oleracea* cv. JZS version 2.0 (Cai *et al.* 2020) were downloaded from the BRAD database (Chen *et al.* 2022). Reads were trimmed by removing low-quality reads and unpaired reads using TRIMMOMATIC version 0.39 (Bolger *et al.* 2014). Subsequently, reads were mapped to their respective reference genomes using bwa-mem (Li and Durbin 2010). Uniquely and high-quality mapping reads were selected by "samtools view -q 20" (-b: output is bam files, -q 20, mapping quality of phred score gives a 99% probability the mapping is correct) (Danecek *et al.* 2021). The depth of each base pair was obtained by "samtools depth -a" (-a all sites). SNP calling was performed using bcftools mpileup and filtered for a minimum quality of 30 and a minimum read depth of 10 using vcftools (Danecek *et al.* 2011), restricted to meiosis gene positions (Supplementary File 5). SNP annotation was performed using CooVar (Vergara *et al.* 2012).

Detection of CNVs in parental *B. rapa* and *B. oleracea* lines

Since protein coding genes are more conserved and less repetitive than other parts of the genome, the detection of CNVs was carried out only for gene coding regions. The median sequencing depth of each gene (based on gene annotation) was calculated. These gene depths were then normalized by dividing by the mean depth of all genes. As our lines are not the same genotypes as the reference genome, there was probably some mapping bias. If all lines from 1 parent species showed low (<0.5-fold relative to mean coverage) or high (>1.5-fold relative to mean coverage) mapping rates, these genes were excluded from the analysis. To avoid uneven distribution of sequencing depth along the genome, a sliding window was calculated for the median depth of 40 genes. Relative read coverage for the median depth of 40 genes was carried out for each of the sequenced *B. rapa* and *B. oleracea* genotypes (Supplementary Figs. 3 and 4).

Identification of meiosis gene candidates in resynthesized *B. napus*

A list of genes annotated as having functions in meiosis was established according to *Arabidopsis* gene annotation (TAIR) and additional published *Brassica* information (Lloyd *et al.* 2014). BLAST (Altschul *et al.* 1990) using an *E*-value cutoff of 10^{-50} was used to pull out meiosis gene copies based on sequence homology to *Arabidopsis* homologs in the *B. rapa* 'Chiifu' v2.5 (Cai *et al.* 2017) and *B. oleracea* 'TO1000' v. 2.1 (Parkin *et al.* 2014) reference genome assemblies. SNPs within these meiosis genes within the 5 *B. oleracea* genotypes (C34, C36, C37, C46, and C47) and 8 *B. rapa* genotypes (A4, A8, A9, A13, A16, and A19, excluding A6 and A7 which were found to be heterozygous) were analyzed using the following steps. Firstly, the SNP data of *B. oleracea/B. rapa* parents were read in. In the next step, all noninformative SNPs were removed: if 1 line had "missing information," this SNP was excluded. Gene haplotypes were obtained from the SNP information. We then inferred the allelic state of the S_1 resynthesized *B. napus* lines by combining the respective parents and subsequently matched these to the phenotype data (fertility, CNVs). Next, one-way ANOVA was used to test for significant differences between haplotypes and total CNVs as well as total seed set. The *P*-values were corrected using the false discovery rate (FDR) test. Subsequently, Fisher's exact test for count data was used to check for significant differences between putatively "stable" and "unstable" lines.

Statistical analysis

Genotypic effects on fertility (self-pollinated seeds and seeds per 10 pods) and genome stability (as measured by number of CNVs) were tested for associations with alleles inherited from either *B. rapa* or *B. oleracea* parent or with the interaction between the 2 in the resynthesized lines. One-way ANOVA was used to test for significant differences in means followed by Tukey's Honest Significant Differences (HSD) test to assess differences between parent *B. rapa* and *B. oleracea* genotype groups using R v. 3.6.3 (The R Team for Statistical Computing).

Results

Purity of S_1 resynthesized *B. napus* lines

SNP genotyping was carried out for all 41 resynthesized *B. napus* genotypes (123 individuals). All individual lines were homozygous and identical in allele inheritance to other individuals in the same progeny set, as expected. However, we observed unexpected differences in allele inheritance between progeny sets with the same parental lines C46, C49, and A19. For progeny sets sharing *B. oleracea* parent line C46, segregating regions were observed on chromosomes C06 (~1.4 Mb) and C08 (2.4 Mb). Progeny sets with

parent C49 also showed segregation on chromosomes C01 (~1.1 Mb) and C04 (0.3 Mb). A 1 Mb region on chromosome A04 was also segregating between progeny sets A19C37, A19C47, and A19C49. Each of C46, C49, and A19 was homozygous inbred lines, rather than doubled haploid; this is likely the origin of the small regions of allelic heterozygosity hypothesized to be present in these progenitor genotypes.

We also observed large differences in A genome allele inheritance between progeny sets of resynthesized lines with *B. rapa* A6 and A7 as A genome parents: lines were homozygous within progeny sets but showed inheritance of different alleles from the A6 and A7 parents between progeny sets. Hence, parent genotypes A6 and A7 were likely actually heterozygous instead of homozygous as expected, explaining why the A genomes of different resynthesized genotype combinations with A6 and A7 *B. rapa* parents were not all the same (Supplementary Fig. 1). Consequently, we renamed all resynthesized *B. napus* lines which had A6 or A7 *B. rapa* parents with codes represented in the form "A6xC1" or "A7xCn," where "x" constitutes a letter from a to f representing a genetically different parent *B. rapa* of that progeny set and "Cn" represents the *B. oleracea* parent genotype (C = *B. oleracea* and *n* = the genotype number). Other progeny sets with *B. rapa* parental genotypes A4, A8, A9, A13, and A16 as well as *B. oleracea* genotypes C34, C36, C37, C38, and C42 (Supplementary File 2) were completely homozygous, as no continuous blocks of heterozygosity and/or allele segregation between progeny sets were observed in these lines.

Resynthesized *B. napus* lines show comparable fertility to parent *B. rapa* and *B. oleracea* genotypes

Total self-pollinated seeds per single plant in the resynthesized lines ranged from 1 to 2,067 (mean 445) (Supplementary Fig. 5a and File 6), with a mean of 45 seeds per 10 pods (range 0–148) (Supplementary Fig. 5b). Resynthesized lines also showed average pollen viability of 81% (Supplementary Fig. 6). Average number of self-pollinated seeds in resynthesized lines was higher compared to *B. rapa* and *B. oleracea* parental genotypes (Fig. 2). Average number of seeds per 10 pods and average number of self-pollinated seeds were moderately highly correlated (r = 0.68) (Supplementary Fig. 7), although there was no significant correlation between pollen viability and either of the seed fertility measures (Supplementary Fig. 8a and b).

Interactions between *B. rapa* and *B. oleracea* parent genotypes affected fertility

Resynthesized *B. napus* lines were assessed in order to detect whether maternal (*B. rapa*) or paternal (*B. oleracea*) genotypes independently influence fertility (total number of self-pollinated seeds and seeds per 10 pods). The total number of self-pollinated seeds produced was significantly affected by *B. rapa* parent genotype (ANOVA, P = 0.000539) (Supplementary Fig. 9a) but not by *B. oleracea* parent genotype (Supplementary Fig. 9b). Neither *B. rapa* nor *B. oleracea* parent genotype independently affected seeds per 10 pods (Supplementary Fig. 10a and b). However, a significant interaction effect (1-way ANOVA, P = 5.97e−05, Tukey's HSD test P < 0.05) was observed for the combination of *B. rapa* and *B. oleracea* parent genotypes on the total number of self-pollinated seeds produced in *B. napus* resynthesized lines based on our linear model (Supplementary Table 1).

Frequent CNVs detected in resynthesized *B. napus* lines

Copy number variants (deletions, reduced copy numbers, and higher copy numbers) were detected at a high frequency across the A and C genomes in the resynthesized *B. napus* lines (Fig. 3). The total number of CNVs detected varied widely between resynthesized *B. napus* individuals (Supplementary Fig. 11 and File 7). No CNVs (>0.5Mb) observed in the resynthesized *B. napus* lines appeared to be inherited from their A or C genome parents.

B. rapa and *B. oleracea* parent genotypes interact to affect genome stability (number of copy number variants) in resynthesized rapeseed lines

Although parent *B. rapa* and *B. oleracea* genotypes independently had no significant effect on the number of CNVs, the interaction between the 2 parent genotypes was significant, such that there were significant differences between resynthesized lines (ANOVA, P = 3.15e−06; Fig. 4; Tukey's HSD test P < 0.05, Supplementary Table 2). Hence, the number of CNVs detected was affected by different cross combinations of *B. rapa* and *B. oleracea* parent genotypes based on our linear model.

The relationship between genome stability (as measured by number of CNVs) and fertility was assessed in the resynthesized *B. napus* individuals. Total number of copy number variants per individual was not significantly correlated with fertility as measured by seeds per 10 pods or pollen viability in resynthesized *B. napus* individuals. A relatively weak significant relationship (Spearman rank correlation = −0.24, P = 0.04) was observed between total number of copy number variants per individual and total seed set per individual. This indicates that other factors apart from genome stability also contribute to fertility in resynthesized lines.

Allelic state of resynthesized S_1 *B. napus* lines predicted by estimating CNVs

CNVs showed a relatively continuous distribution from low to high across the resynthesized *B. napus* lines. However, in order to carry out further analyses, 19 resynthesized *B. napus* lines, excluding cross combinations with heterozygous A6 and A7 parents, were classified into stable (more stable), intermediate, and unstable (less stable) using the following criteria and process. Firstly, we undertook pairwise comparisons of CNV data to determine which resynthesized lines were significantly different from each other (Tukey's HSD test P < 0.05, Supplementary Table 2). Two groups were established which were significantly different from each other in numbers of CNVs: these lines were classified as either putatively "stable" (low numbers of CNVs) or putatively "unstable" (high numbers of CNVs) while lines which were not significantly different from any other line were classified as "intermediate." Based on this, resynthesized lines with average number of CNVs below 6 were classified into putatively "stable" (4 combinations), from 6 to 10 putatively "intermediate" (11 combinations), and above 10 as putatively "unstable" (4 combinations) (Table 1).

Allelic variation in meiosis genes is associated with number of CNVs

Eight parent accessions of *B. rapa* (A4, A6, A7, A8, A9, A13, A16, and A19) and 5 parent accessions of *B. oleracea* (C34, C36, C37, C46, and C47) were resequenced. However, *B. rapa* A6 and A7 accessions were found to be heterozygous and were subsequently taken out of the analysis. In the next step, the allelic variation was analyzed in a list of meiosis gene homologs. A total of 3,689 SNPs in *B. rapa* meiosis genes were detected, of which 832 were nonsynonymous and no splice variants were detected (Supplementary File 7a). In *B. oleracea* meiosis genes, 2,549 SNPs were detected, of which 729 were nonsynonymous, 4 were splice variants, and 3 were stop co- don gains (Supplementary File 7b). Moreover, CNVs in meiosis

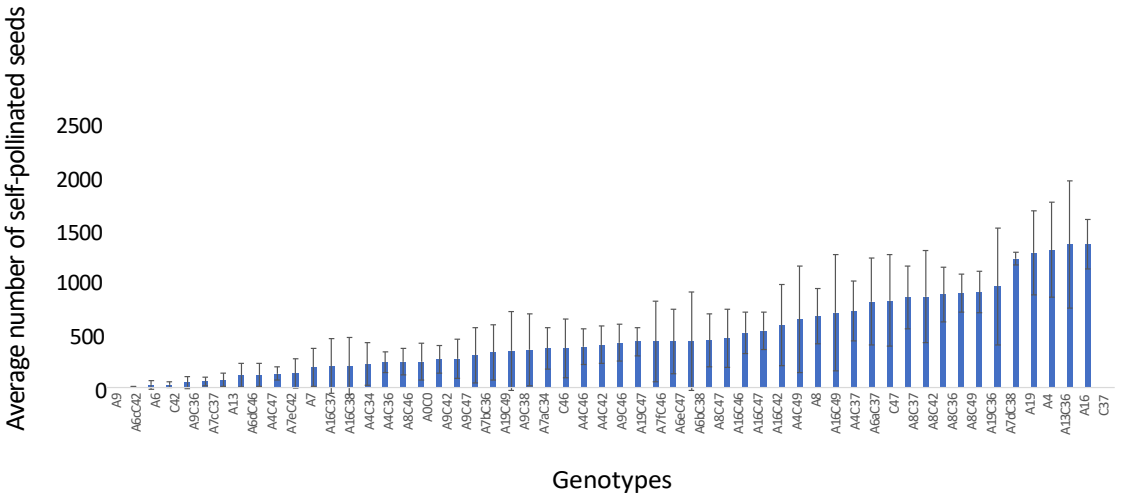

Fig. 2. Fertility of resynthesized *B. napus* lines measured by the average number of self-pollinated seeds was compared to progenitor *B. rapa* (A4, A6, A7, A8, A9, A13, A16, and A19) and *B. oleracea* (C34, C36, C37, C42, C46, and C47) genotypes. Resynthesized lines are indicated by the parent combination in the form AnCn or AnxCn. "x" constitutes a letter from a to f representing a genetically different parent *B. rapa* of that progeny set, as *B. rapa* genotypes A6 and A7 were found to be heterozygous.

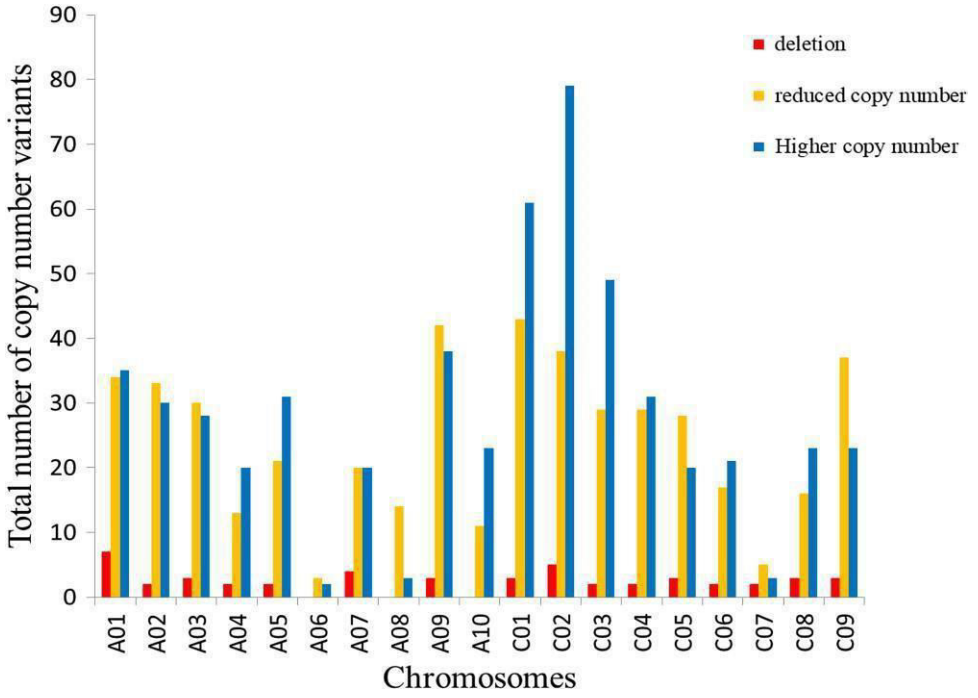

Fig. 3. Genome-wide distribution of CNVs detected in resynthesized *Brassica napus* individuals. Deletions (zero copies of a chromosome region) are indicated in red, reduced copy (1 copy of a chromosome region) in yellow, and higher copy number (3 or more copies of a chromosome region) in blue.

genes were detected by analyzing coverage. It was found that 96 of the 197 *B. rapa* meiosis gene copies predicted from the reference genome carried a deletion in at least 1 accession, out of which 2 were deleted in all 8 accessions, and 90 gene copies carried a duplication (Supplementary File 7c). In *B. oleracea*, 33 gene copies out of 193 were deleted in at least 1 accession, 1 of them in all accessions, and 57 were duplicated (Supplementary File 7d).

From these data, the allelic state of the S_1 *B. napus* resynthesized lines by combining the respective parents was inferred (Supplementary File 7e and f) from which the list of putative meiosis genes candidates was pulled (Supplementary File 8a–d).

Phenotypic data for 19 cross combinations (excluding combinations with heterozygous A6 and A7 parents) which could be tested in the greenhouse were used, and total CNV counts genome-wide classified lines into putatively "stable" (4 combinations), "intermediate" (11 combinations), "unstable" (4 combinations), and "missing" (11 combinations) (Table 1). In the next step, meiosis candidate genes were selected using the following criteria (Supplementary File 8a–e): firstly, significant associations of number of CNVs with meiosis gene haplotypes after FDR correction (Supplementary File 8b and e); secondly, presence of putatively harmful mutations in meiosis gene haplotypes which fulfill

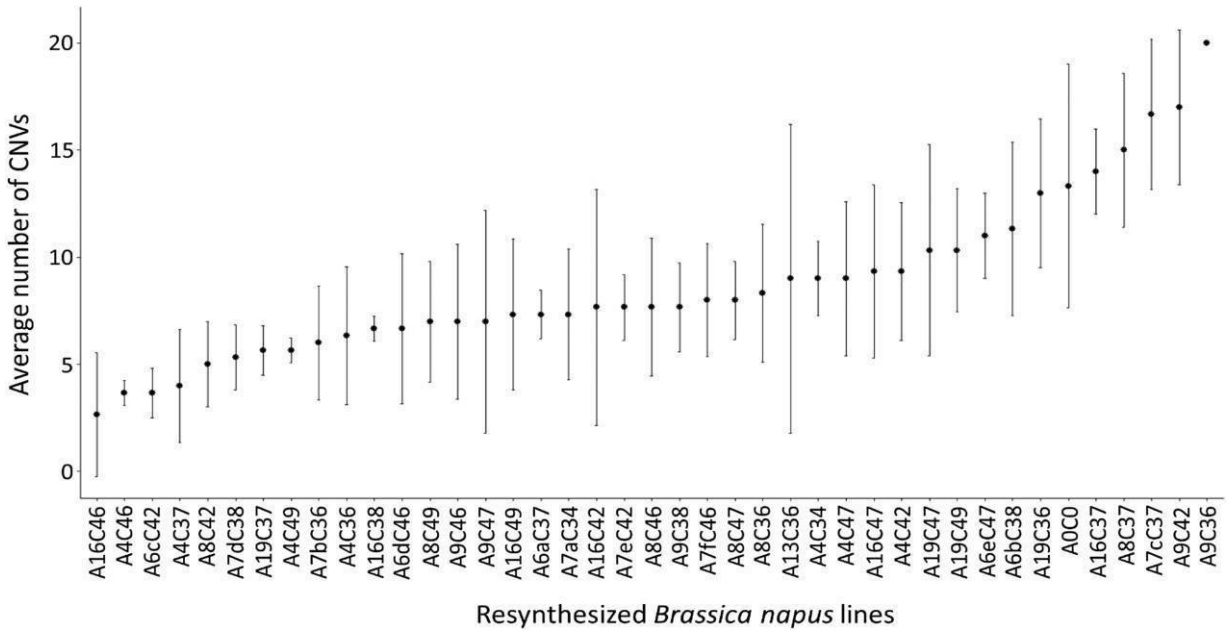

Fig. 4. Average number of CNVs in resynthesized *B. napus* (rapeseed) lines comprising different genotype combinations (3 individuals per line). Parent genotypes of *B. rapa* are A4, A6, A7, A8, A9, A13, A16, and A19; parent genotypes of *B. oleracea* are C34, C36, C37, C38, C42, C46, C47, and C49; and synthetic rapeseed lines are indicated by the parent combination in the form AnCn or AnxCn. "x" constitutes a letter from a to f representing a genetically different parent *B. rapa* of that progeny set, as *B. rapa* genotypes A6 and A7 were found to be heterozygous. Statistical comparisons between lines can be found in Supplementary Table 2.

criterion 1 (nonconservative missense codon, stop codon gain var- iants, and/or splice variants) (Supplementary File 8b and e); and thirdly, putative gene function in meiosis related to DNA or double-strand break repair, effects on meiotic crossover, or effects on homoeologous recombination (Supplementary File 8e).

Using these 3 criteria, we identified 13 putative meiosis genes from the *B. oleracea* C genome parents used to produce the resynthesized *B. napus* lines (Table 2). Of these, *RPA1C*, *MSH2*, and *RECQ4B* also showed presence of either a stop codon or a splice variant in at least 1 gene copy. *RPA1C* gene copies carried 2 nonconservative missense codons and 1 stop codon gain. *MSH2* carried 1 stop codon gain and 1 splice donor variant, while *RECQ4B* showed 4 missense codons, 1 stop codon gain variant, and 1 splice acceptor variant. Other putative candidate genes with significant CNV association with haplotypes and carrying at least 1 nonconservative missense codon were *BRCA*, *ATR*, *RAD51C*, *MLH3*, *RECQ4A*, *SDS*, *RAD51*, *BLAP75/RMI1*, *SYN1/DIF1/REC8*, and *AtGR1/COM1* (Table 2). In the *B. rapa* parents, none of the gene haplotypes fulfilled the criterion of significant (after FDR correction) association with total CNV number, which was our most important criterion for the selection of putative meiosis gene candidates. So these genes from *B. rapa* parents (Supplementary File 8c and d) were not considered as interesting meiosis gene candidates.

Genetic analyses of resynthesized *B. napus* lines

Analysis of the genetic background of the parental lines used in this study showed that maternal *B. rapa* var. *trilocularis* was the parent for all stable resynthesized lines (4 out of 4 considered as putatively stable), although this subspecies also contributed to intermediate (8 out of 11) and unstable lines (3 out of 4) (Table 1). Resynthesized cross combinations with C46 (*B. oleracea* var. *alboglabra*) as paternal *B. oleracea* parent were also either putatively stable or intermediate (Table 1). Although it is not possible to draw statistically significant conclusions from these results in the current study, further investigation of this association may be warranted in future.

Discussion

In this study, we aimed to test the hypothesis that allelic variants inherited from parental genotypes of *B. rapa* and *B. oleracea* would affect meiosis in newly resynthesized rapeseed lines. To this end, we analyzed relative genome stability (as measured by copy number variants) and measured fertility using self-pollinated seed set, seeds per 10 pods, and pollen viability in a set of lines of resynthesized *B. napus* with common parent genotypes after 1 generation of self-pollination as well as screened for variants of meiosis candidate genes possibly affecting genome stability in the lines. Our results show that allelic variants inherited from both diploid *B. rapa* and *B. oleracea* parents interact to affect genome stability and fertility in resynthesized *B. napus* lines. Resynthesized rape- seed lines from different genetic backgrounds also vary signifi- cantly in both fertility and genome stability. We identified 13 putative meiosis candidate genes which were significantly asso- ciated with frequency of copy number variants and which con- tained putatively harmful mutations in meiosis gene haplotypes for further investigation.

Our results show a negative correlation between genome stability (CNVs) and fertility as measured by total seed set ($P = 0.04$, Spearman correlation $r = -0.24$) in resynthesized *B. napus*. In nat- ural *B. napus*, inheritance of unbalanced translocation events in mapping populations was also associated with a fertility penalty (Osborn *et al.* 2003). Negative correlations between chromosome rearrangements and fertility have also been observed in both natural and resynthesized *B. napus* populations (Samans *et al.* 2017). These results support the present study where CNVs were significantly negatively associated with fertility (self-pollinated seed set). However, the detected correlation was low, indicating that

Table 1. Classification of resynthesized *Brassica napus* cross combinations resulting from homozygous parent *B. rapa* and *B. oleracea* genotypes into putatively "stable," putatively "unstable," and putatively "intermediate" by pairwise comparisons and estimation of average numbers of CNVs.

	C34 var bot dh	C36 var bot dh	C37 var bot dh	C46 var alb il	C47 var alb il
A4 var tri il	Intermediate	Intermediate	Stable	Stable	Intermediate
A16 var tri il	—	—	Unstable	Stable	Intermediate
A19 var tri il	—	Unstable	Stable	—	Intermediate
A8 var tri dh	—	Intermediate	Unstable	Intermediate	Intermediate
A9 var olei dh	—	Unstable	—	Intermediate	Intermediate
A13 var pek dh	—	Intermediate	—	—	—

Genotypes without data (no plants) are indicated with "—." Lines are from Abel *et al.* (2005). var olei, *B. rapa* var. *oleifera*; var tri, *B. rapa* var. *trilocularis*; var bot, *B. oleracea* var. *botrytis*; var alb, *B. oleracea* var. *alboglabra*; dh, double haploid; il, inbred lines.

other factors apart from genome stability are contributing to fer- tility in resynthesized *B. napus* lines. As well, we would predict that in translocation heterozygotes (indicated by CNVs where we see 1 or 3 copies of a chromosomal region), both size and relative A–C genome homoeology/chromosomal location of the CNV would have large effects, although we were not able to resolve this level of detail in the current study.

We observed a wide range of genotype-dependent fertility across the resynthesized *B. napus* lines, and some resynthesized lines showed higher fertility than *B. rapa* and *B. oleracea* parental genotypes in our study. Malek *et al.* (2012) detected higher fertility in synthetic *B. napus* compared to its parental *B. rapa* and *B. oleracea* genotypes in terms of the number of seeds per silique, 1000-seed weight, and seed yield per plant, in support of the hypothesis that polyploid crops (interspecific hybrids) often show higher yield levels and outperform their diploid relatives (Sattler *et al.* 2016), highlighting the heterotic potential of resynthesized *B. napus* lines (Abel *et al.* 2005). Rousseau-Gueutin *et al.* (2017) assessed fertility (number of seeds/50 pollinated flowers and number of seeds/50 pods) in both open-pollinated and manually self-pollinated re- synthesized *B. napus* populations and found very low fertility com- pared to natural *B. napus* using both fertility measures. Rousseau-Gueutin *et al.* (2017) hypothesized that self-incompatibility alleles carried by the parental diploid species might have affected the fertility of their hybrids, since different subspecies of *B. rapa* and *B. oleracea* parents had been used to pro- duce the resynthesized *B. napus* population. Many genotypes of *B. rapa* and *B. oleracea* are self-incompatible, a trait genetically con- trolled by the self-incompatibility *S*-locus (Camargo *et al.* 1997; Kimura *et al.* 2002; Kitashiba and Nasrallah 2014), which prevents self-seeds. Self-pollinated seed set varied greatly across our *B. rapa* and *B. oleracea* parents and their progeny, with possible self-incompatibility issues in a few parental genotypes used to pro- duce the resynthesized lines (Fig. 2). One genotype, *B. rapa* A9, was most likely self-incompatible, setting no self-pollinated seed. Interestingly, none of the synthetic combinations with A9 were completely sterile, and some were highly fertile, suggesting the synthetic *B. napus* mostly overcame self-fertilization via recog- nition in the stigma and failed germination of pollen with the same *S*-haplotype as the parent plant. However, self-incompatibility alleles present in some parent genotypes used to produce our resynthesized lines might be responsible for low fer- tility in a few lines (9 genotypes produced <15 seeds). Further in- vestigation would be needed to confirm this.

In this study, most of our resynthesized *B. napus* genotypes averaged >5 CNVs per plant across the A and C genomes. Several studies have shown that chromosomal rearrangements occur frequently in both resynthesized and natural *B. napus* (Pires *et al.* 2004; Udall *et al.* 2005; Leflon *et al.* 2006; Liu *et al.* 2006; Nicolas *et al.* 2007; Chalhoub *et al.* 2014; Guo *et al.* 2016). Homoeologous exchanges have been detected in translocated re- gions (deletion–duplication events) between A and C homoeolo- gous chromosomes in both resynthesized and natural *B. napus*, where we see a gradient of decreasing translocation frequency with decreasing size of homoeologous regions between the A and C genomes (Chalhoub *et al.* 2014; Samans *et al.* 2017; Mason *et al.* 2018). This pattern was also observed in our study. Samans *et al.* (2017) analyzed 52 highly diverse *B. napus* genotypes includ- ing 32 natural and 20 synthetic *B. napus* accessions using whole genome sequencing and detected a greater number and size of genomic rearrangements in synthetic *B. napus* compared to nat- ural accessions as well as more areas with deletions than duplica- tions. We also found fewer deletions (5.6%) than either reduced (1 missing copy) or higher copy number (1 or 2 extra copies as pre- dicted by the pipeline) variants. However, our inability to discrim- inate between 3 or 4 copies (the combined "higher copy number" category) prevents us from making conclusions about the preva- lence of deletions relative to duplications. Also, in contrast to the abovementioned studies, which were all on established lines of *B. napus* or synthetic *B. napus* which had been through many generations of self-pollination, our resynthesized *B. napus* mater- ial has undergone only 1 self-pollination event (2 meioses). Hence, any novel variants which have arisen are unlikely to be "fixed" (homozygous, present in both homologous chromosomes), as the products of a single homoeologous crossover event during meiosis rarely segregate together: 2 recombinant chromatids are produced from 1 homoeologous crossover, but these are usually separated into different gametes in the first meiosis. Subsequently, selection against unbalanced translocations may "fix" these events in self-pollinated progeny, resulting in balanced duplication/deletions.

Our copy number pipeline was not robust enough to efficiently discriminate whether 3 copies of an allele or more copies (4+) were present, leading us to use a combined "higher copy number" cat- egory. Our copy number pipeline may also be overestimating re- duced and higher copy number CNV calls. Failed SNP calls from the 60K *Brassica* SNP chip used possibly lowered the average copy numbers across specific regions, leading to high false posi- tive error rates (Mason *et al.* 2017). In addition, lower signal detec- tion may result from other types of sequence polymorphism other than CNVs, which may also result in false positives as reported by Zmieńko *et al.* (2014). Although both hybridization-based arrays and next-generation sequencing approaches used for the detec- tion of CNVs have different limitations (Zmieńko *et al.* 2014), high-coverage sequencing likely provides a more robust method of call- ing CNVs (Yoon *et al.* 2009). However, obtaining sufficient read depth is still factorially more expensive than calling CNVs from array data (Mason *et al.* 2017).

Table 2. Meiosis gene haplotypes associated with CNV frequencies in resynthesized *B. napus* derived from crosses between *B. rapa* and *B. oleracea* parents.

Candidate genes involved	Chromosome	Location of other copies	*B. oleracea* copies	*A. thaliana* homolog	Type of mutation	Significant CNV association with haplotypes after FDR correction ($P < 0.05$)
RPA1C	C02	C09	*Bo2g127130.1, Bo9g061490.1*	*AT5G45400.1, AT5G45400.1*	Nonconservative missense codon, stop codon gain	0.04, 0.04
MSH2	C06	C06, C03	*Bo6g030570.1, Bo3g071550.1, Bo6g003510.1*	*AT3G18524.1, AT3G18524.1, AT3G18524.1*	Stop codon gain, splice variant donor	$P > 0.05$, 0.03, $P > 0.05$
RECQ4B	C09	—	*Bo9g043460.1*	*AT1G60930.1*	Nonconservative missense codon, stop codon gain, splice acceptor variant	0.04
AtGR1/COM1	C04	—	*Bo4g125630.1*	*AT3G52115.1*	Nonconservative missense codon	0.03
ATR	C04	—	*Bo4g145640.1*	*AT5G40820.1*	Nonconservative missense codon	0.03
BRCA	C01	C01	*Bo1g023820.1, Bo3g001340.1*	*AT4G21070.1, AT5G01630.1*	Nonconservative missense codon	0.03, 0.04
RAD51C	C04	—	*Bo4g038470.1*	*AT2G45280.1*	Nonconservative missense codon	0.04
MLH3	C07	—	*Bo7g117460.1*	*AT4G35520.1*	Nonconservative missense codon	0.03
RECQ4A	C08	C08	*Bo8g059730.1, Bo8g109200.1*	*AT1G10930.1, AT1G10930.1*	Nonconservative missense codon	0.03, 0.03
SDS	C08	C05	*Bo8g066230.1, Bo8g106580.1, Bo5g019980.1*	*AT1G14750.1, AT1G14750.1, AT1G14750.1*	Nonconservative missense codon	0.04, 0.03, 0.03
RAD51	C09	C03	*Bo9g151450.1, Bo3g015380.1*	*AT5G20850.1, AT5G20850.1*	Nonconservative missense codon	0.04, 0.03
BLAP75/RMI1	C09	—	*Bo9g017740.1*	*AT5G63540.1*	Nonconservative missense codon	0.03
SYN1/DIF1/REC8	C09	—	*Bo9g177100.1*	*AT5G05490.1*	Nonconservative missense codon	0.04

We observed that different cross combinations of *B. rapa* and *B. oleracea* genotypes significantly affect genome stability (as measured by number of CNVs after self-pollination; 2 meiosis events) based on our linear model. This observation is likely due to interactions between specific allelic variants from the parent genotypes which influenced genome stability in the resynthesized lines. Hypothetically, different allelic variants present in *B. rapa* and *B. oleracea* may be present but only have an effect on meiosis after combination of these 2 genomes in a single cell. Such an effect was observed for genetic locus *PrBn* (Jenczewski *et al.* 2003), which clearly segregated allohaploid *B. napus* (2n = AC) into "high-pairing" and "low-pairing" phenotypes based on meiotic behavior (specifically frequency of A–C chromosome pairing observed at metaphase I), but which was found to have no effect on meiosis in established allopolyploid *B. napus* (2n = AACC). As well, *B. rapa* and *B. oleracea* are mesopolyploid genomes which show a triplicated genome structure relative to Brassicaceae relative *Arabidopsis* (The *Brassica rapa* Genome Sequencing Project Consortium *et al.* 2011; Liu *et al.* 2014; Parkin *et al.* 2014): although many meiosis gene copies are thought to have returned to a functional diploid state with only a single working gene copy (Lloyd *et al.* 2014), the extent of this pseudogenization and whether it is consistent across all genotypes (particularly those used in our study) is unknown. Therefore, another source of genetic variation is predicted to be the number of working meiosis gene copies across both parental genomes (*B. rapa* and *B. oleracea*), which may explain the observed genotypic interaction effects we found (meiotic effect as a sum of working meiosis gene copies in both genomes). In support of this hypothesis, Gonzalo *et al.* (2019) found functional compensation of meiotic phenotype was conferred by only a single working meiosis gene copy in either of the A or C genomes in knockout lines for *MSH4*. Additionally, Gaebelein *et al.* (2019) found significant QTL for fertility (as a proxy for meiotic stability) harboring different copies of the same meiosis gene in 2 genomic locations in a synthetic *Brassica* allohexaploid population. Gaeta *et al.* (2007) suggested that *B. napus* might have initially been unstable, but that alleles responsible for genetic control of meiosis inherited from 1 or both diploid progenitors may have been selected for over time, possibly by conferring improved seed set. In *A. arenosa*, selection of specific alleles of meiosis genes seems to be responsible for reduced crossover frequency, result- ing in meiotic stability in the polyploid (Yant *et al.* 2013). Recently, allelic variants of *ASY1* and *ASY3* in particular were found to reduce multivalent frequencies and help regulate mei- osis in polyploid *A. arenosa* (Morgan *et al.* 2020), and introgression of meiosis gene alleles from *A. arenosa* was found to help stabilize tetraploid *Arabidopsis lyrata* (Marburger *et al.* 2019). Similarly, selection of genetic variants at preexisting loci may have contributed to form stable meiosis in ancient polyploid *Brassica* (Lloyd *et al.* 2014).

We presented a summary of 13 putative meiosis gene candidates which show significant CNV association and presence of putatively harmful mutation in meiosis gene haplotypes as well as putative gene function in meiosis related to DNA or double-strand break repair, effects on meiotic crossover, or suppression of homoeologous recombination. Of the 13 genes, 3 are of special interest due to the presence of stop codons or splice variants in at least 1 copy: *RPA1C*, *MSH2*, and *RECQ4B*. Due to the functional redundancy of many meiosis genes within *Brassica* species (Lloyd *et al.* 2014), particularly in the 2n = AACC allopolyploids (Gonzalo *et al.* 2019; Higgins *et al.* 2021), loss-of-function gene mutations are excellent candidates for major phenotypic effects on meiosis.

Replication protein A (*RPA*) is a eukaryotic, single-stranded DNA-binding protein made up of 3 subunits *RPA1*, *RPA2*, and *RPA3* and plays important roles in almost all DNA metabolic pathways including *S*-phase genome replication, DNA recombination, and DNA excision repair (Aklilu *et al.* 2014). *RPA1C* has been shown to promote homologous recombination in early meiosis, which may relate to an as-yet unknown role in regulation of nonhomologous recombination, and interactions between *RPA1C* and *RPA1E* are primarily responsible for DNA repair in *Arabidopsis thaliana* (Aklilu *et al.* 2014; Aklilu and Culligan 2016). In rice, *RPA1C* is shown to be required for ~79% of chiasma formation, and the *RPA* complex comprising *RPA1C* and *RPA2C* is required to promote meiotic crossovers (Li *et al.* 2013). In *B. napus*, *RPA1C* was found within the *BnaA9* QTL region responsible for the prevention of homoeologous chromosome pairing (Higgins *et al.* 2021). In the present study, we also found 1 copy of *RPA1C* on chromosome A09 as well as 2 copies of *RPA1C* on chromosomes C02 and C09 from the *B. oleracea* parent of resynthesized *B. napus*. The 2 C genome copies were both significantly associated with CNVs and fertility, and radical SNP mutations were observed in both C02 and C09 copies while a stop codon gene variant was predicted in the C02 copy. Hence, even though different copies of this gene were implicated in our study relative to the study of Higgins *et al.* (2021) (in the C genome rather than the A genome), we suggest that these gene copies are all excellent candidates for future functional validation (e.g. via characterization of knockout mutants and via complementation analysis using genetic transformation to see if knockin of this gene restores the observed phenotype).

MutS is an ATPase involved in mismatch recognition, a potentially key element for discrimination between homologous (more similar) and homoeologous (less similar) chromosome sequences during meiosis, with 4 MutS homologs identified in *Arabidopsis* (*AtMSH2*, *AtMSH3*, *AtMSH6*, and *AtMSH7*) on the basis of their sequence conservation (Emmanuel *et al.* 2006). The *MSH2* protein regulates meiotic recombination during prophase 1, thereby functioning in a pro-crossover role in regions of higher sequence diversity in *A. thaliana*. *AtMSH2* has also been shown to have an antirecombination meiotic effect in *A. thaliana* (Emmanuel *et al.* 2006). *MSH2* was found in the QTL interval underlying fertility on chromosome C3 in *Brassica* allohexaploids derived from a cross (*B. napus* × *B. carinata*) × *B. juncea* (Gaebelein *et al.* 2019). Here, we found 3 gene copies of *MSH2* in the *B. oleracea* parent genome: 2 copies on chromosome C06 and 1 copy on C03. Although 1 of the gene copies on C06 showed no significant association with CNV number, a stop codon gain variant and a splice variant donor were observed as allelic variants of this gene copy. However, the other C06 copy was not significantly associated with fertility or CNV traits, with no SNP mutations observed. Another gene copy on chromosome C03 was significantly associated with CNV and total seed set and contained a missense codon.

RecQ helicases are involved in the processing of DNA structures arising during replication, recombination, and repair throughout all kingdoms of life (Hartung *et al.* 2007). Seven different *RecQ* genes are present in *Arabidopsis*. Among them are 2 paralogs, *RECQ4A* and *RECQ4B*, which arose as a result of a recent duplication and which are nearly 70% identical on a protein level (Hartung *et al.* 2007; Schröpfer *et al.* 2014). In *Arabidopsis*, *RECQ4A* and *RECQ4B* have both been shown to limit crossovers (Fernandes *et al.* 2018; Serra *et al.* 2018), which may assist in reducing nonhomologous recombination frequency. However, an earlier study showed that *AtRECQ4B* is specifically required to promote but not to limit crossovers, a role which is different from all other known eukaryotic *RecQ* homologs (Hartung *et al.* 2007). de Maagd *et al.* (2020) investigated the role of tomato *RecQ4* on crossover formation in an interspecific cross between cultivated tomato and 1 of its wild relatives, and observed a 1.53-fold increase of ring bivalents, suggesting a less important role in limiting crossover compared to *Arabidopsis*. Here, we found *RECQ4B* on chromosome C09 in *B. oleracea* used to produce our resynthesized *B. napus* interspecific cross. *RECQ4B* was significantly associated with CNV numbers and showed predicted radical SNP mutations with a potential harmful effect on protein function, as observed by a stop codon gain variant, as well as a splice acceptor variant. Two copies of *RECQ4A*, which is the other paralog of *RECQ4B*, were found on C08. Both copies were also significantly associated with CNVs and seed set, with radical SNP mutations as indicated by the presence of nonconservative missense codons. Based on the literature, we would perhaps expect this gene to play a role in reducing nonhomologous crossovers via reduction of total crossover frequency, rather than in allowing discrimination between homologous and nonhomologous chromosomes, and hence to play a more minor role in meiotic stabilization in *Brassica*.

Genetic variation in meiosis genes in general may cause large effects on genome stability in different plant lineages (Addo Nyarko and Mason 2022). Although *B. napus* is not thought to have undergone detectable gene fractionation since formation (Chalhoub *et al.* 2014), knockout of 1 existing *MSH4* gene copy was shown to help prevent nonhomologous chromosome pairing in *B. napus* (Gonzalo *et al.* 2019), supporting the idea that loss of functional meiosis gene copies in mesopolyploids *B. rapa* and *B. oleracea* may also then contribute to formation of allopolyploids with higher meiotic stability. We could not identify any interesting meiosis gene candidates from the *B. rapa* parent genotypes based on our analysis, most likely due to the small numbers of genotypes in our study. We have identified putative meiosis genes present in the diploid *B. oleracea* progenitor genotypes used to produce our resynthesized *B. napus* lines, some of which were present in more than 1 copy. Meiosis gene copies have been shown to be under strict control, with most genes returning rapidly to single copies (Lloyd *et al.* 2014), presumably to avoid meiotic abnormalities caused by the retention of several gene copies following polyploidization. However, allelic variants of meiosis genes which are only present in a few copies could potentially have an impact on genome stability and/or fertility of resynthesized *B. napus*. Hence, the respective allelic variants of the putative meiosis genes identified are putatively good candidates for the variation in copy number observed in our study. *B. napus* itself appears to be too young (<10,000 years) to have undergone any major gene fractionation: almost all (if not all) A and C genome gene copies in *B. napus* are still intact (and expressed similarly) relative to progenitor *B. rapa* and *B. oleracea* subgenomes (Chalhoub *et al.* 2014). Limited subgenome differentiation or specialization of A and C genome copies has also been observed with regard to gene expression in synthetic *B. napus* (before meiosis) relative to its parent genotypes: although differences between the subgenomes exist, these appear to be mainly inherited directly from the progenitor diploids (e.g. Bird *et al.* 2021; reviewed by Katche and Mason 2023). However, *B. rapa* and *B. oleracea* are themselves mesopolyploids, and not all meiosis genes have been reduced to single copy in these species (Lloyd *et al.* 2014). Gene copies within these mesopolyploid diploid genomes also show major differentiation in terms of gene expression and function, with clear differences between subgenomes (The *Brassica rapa* Genome Sequencing Project Consortium *et al.* 2011; Parkin *et al.* 2014; Cheng *et al.* 2016). Hence, *B. rapa* and *B. oleracea* may contain allelic variants including loss-of-function

mutations which could conceivably affect meiosis in resynthesized *B. napus*.

Our results show that some resynthesized lines are more genomically stable and fertile than others and suggest that allelic variation present in both of the diploid parents interacts to affect the chance of chromosome rearrangement and CNV events, but that the presence of such events may not always be detrimental to fertility in resynthesized *B. napus* lines. Our study suggests meiotic stability in *B. napus* arose via selection of allelic variants from its diploid progenitor species and provides information that will be useful for breeders aiming to use resynthesized lines in breeding programs. The production of genomically stable resynthesized *B. napus* lines might be useful in the future as a germplasm resource to broaden the limited genetic diversity of established *B. napus* cultivars or for hybrid breeding (Abel *et al.* 2005).

Data availability

The data that support the findings of this study are available in the supplementary material of this article. Sequencing data generated in this project are available under BioProject accession code PRJNA724876 from the National Center for Biotechnology Information (NCBI).

Supplemental material available at G3 online.

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Conflicts of interest

The authors declare no conflict of interest.

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5.0 Discussion

5.1 Overview and scientific contribution of this thesis

In this thesis, I investigated the fertility and genome stability of early and later generation resynthesized Brassica napus lines produced by crosses between *Brassica rapa* and *Brassica oleracea* as well as between *Brassica rapa* and wild C genome species. The main goal of this thesis was to characterize a diverse set of resynthesized lines for homozygosity (purity), fertility and genome stability by using copy number variation as a proxy as well as to test the hypothesis that specific allelic variants inherited from diploid progenitors conferred meiotic stability on established *B. napus*. The possibility of producing genomically stable resynthesized *B. napus* genotypes presents an invaluable genetic resource for farmers for direct use in rapeseed breeding, and for the introgression of agronomically important traits. This is particularly necessary to increase the genetic diversity of the current rapeseed gene pool which has already been eroded as a result of intensive breeding effort to produce high quality rapeseed oil with low glucosinolate and low erucic acid content. Most resynthesized *B. napus* which have been produced have been reported to be meiotically unstable and infertile, similar to the result of this thesis where most lines show high number of copy number variants indicative of meiotic instability. However, in this thesis we observed eight putatively stable resynthesized lines, as indicated by the presence of low number of copy number variants, suggesting that it is possible to select genomically stable and fertile resynthesized *B. napus* for breeding purposes. A high rate of contamination (66%) because of heterozygosity (outcrossing with unknown parents) was observed in the resynthesized *B. napus* analysed especially in older materials. The results of this thesis suggest that it is important to screen for homozygosity before resynthesized lines are used for polyploidy research. I identified 13 putative meiosis gene candidates which were significantly associated with copy number variants, and which contained putatively harmful mutations in meiosis gene haplotypes. This result supports the hypothesis that specific allelic variants from parental genotypes affect genome stability and fertility in resynthesized *B. napus*. Therefore, the outcomes of this study suggest that allelic variants from diploid progenitors conferred meiotic stability in *B. napus*. This thesis also shows that some resynthesized *B. napus* are stable and fertile and can be maintained for many generations as highly useful germplasm resources for breeding and research.

5.2 Fertility and genome stability of resynthesized *B. napus*

Fertility is one major challenge faced by sexually reproducing neopolyploids that hinders their successful establishment (Rousseau-Gueutin et al. 2017). This is usually as a result of meiotic instability which has been shown to be prevalent in neopolyploids and newly created interspecific hybrids (Mason et al. 2010; Szadkowski et al. 2010; Xiong et al. 2011; Chester et al. 2013; Grandont et al. 2014). Rousseau-Gueutin et al. (2017) assessed fertility in both open pollinated and self-fertilized early generation resynthesized lines (S_1- S_3), and observed lower fertility compared to *B. napus* cultivars, with fertility significantly reduced over subsequent generations. The reduction in fertility was suggested to be influenced by the origin of the diploid *B. rapa* and *B. oleracea* parent genotypes used to produce the hybrids. Differences in fertility were observed in the two resynthesized population analysed which belong to two different subspecies, with one population being less fertile compared to the other (Rousseau-Gueutin et al. 2017). Malek et al. (2012) observed higher fertility in resynthesized *B. napus* lines compared to *B. rapa* and *B. oleracea* parent genotypes. Xiong et al. (2011) assessed pollen viability and seed set in later (S10-S11) generation resynthesized *B. napus* lines produced by crossing one genotype each for *B. rapa* and *B. oleracea*, and also observed reduced fertility with subsequent generations. The observation of reduced fertility compared to established *B. napus* in the above previous studies parallels what was observed in the fertility assessment of early generation resynthesized lines (Chapter 4). However, a few resynthesized lines in later generations showed comparable fertility to *B. napus* cultivars, and higher fertility than *B. rapa* and *B. oleracea* genotypes (Chapter 3). Improved seed set was observed after the first two generations in our study (Chapter 3) in contrast to other studies in resynthesized *B. napus*. This difference might be as a result of differences in the number of genotypes compared to other study as a large number of resynthesized lines produced from diverse *B. rapa* and *B. oleracea* parents were investigated in this thesis compared to previous studies. Similar to the results obtained here, other studies in different types of *Brassica* hybrids also observed increased fertility over subsequent generations (Tian et al. 2010; Gaebelein et al. 2019; Katche et al. 2021). Early generation resynthesized lines with poor fertility most likely did not survive to subsequent generations, which probably means that fertile early generation genotypes became more fertile after the first two generations compared to lines in their S_1 and S_2 generations in our study.

Chromosome rearrangement indicative of genome instability was observed in resynthesized *B. napus* lines analysed irrespective of their level of fertility (Xiong et al. 2011). However, resynthesized lines generated were propagated and maintained for ten rounds of self pollination regardless of genome instability and poor fertility (Xiong et al. 2011). Although how established *B. napus* attained genome stability is still being studied, results obtained from studies with resynthesized lines showing varied fertility and different levels of genome instability suggest that natural selection against poorly fertile and sterile individuals, and selection for highly fertile and genomically stable plants contributed to meiotically stable natural *B. napus* cultivars (Xiong et al. 2011).

Copy number variation (CNV) refers to the presence of DNA sequences usually larger than 1 Kb in size which differ in the number of copies between individuals or populations of the same species (Schiessl et al. 2019). CNVs arise as a result of several mechanisms, including non-allelic homologous recombination occuring in DNA regions of high sequence similarity between subgenomes which share common ancestry (Zmieńko et al. 2014). CNVs may also arise following whole genome duplication events resulting in a change in gene copy number due to subsequent deletions in either of the subgenomes (Lye and Purugganan 2019). Copy number variants (CNVs) play a major role in agronomically important traits in plants (Dolatabadian et al. 2017; Schiessl et al. 2017). CNV is an important source of genetic variation during domestication, and has the potential to be used as sources of useful traits which can be used to improve cultivars from crop wild relatives or landraces (Lye and Purugganan 2019). Copy number variation as a result of homoeologous exchange was implicated in specific *B. napus* copies of Flowering Locus C on chromosome A2 in both resynthesized and natural *B. napus* cultivars (Chalhoub et al. 2014). In meiotically stable *B. napus* cultivars, homoeologous recombination caused by accumulation of CNVs is rarely observed in contrast to newly synthesized *B. napus* hybrids (Chalhoub et al. 2014; Xiong et al. 2021). Hence, the number of CNVs present in an individual or hybrid population is a measure of their genome stability and fertility. Ferreira de Carvalho et al. (2021) analysed resynthesized *B. napus* lines produced by repeatedly selecting for euploid individuals up to eight generations using single seed descent and observed a high negative correlation between the presence of homoeologous chromosome rearrangements and seed yield. Here, we observed negative correlations between copy number variation and fertility as measured by self-pollinated seeds, seeds per ten pods, and pollen viability in both studies produced by this thesis. The accumulation of large number of CNVs were found to significantly associate with reduced fertility (Chapter 3). Based on this result we

can assume that the presence of higher frequencies of CNV in some regions of chromosomes might be detrimental to genome stability and/or fertility, although some CNVs could be a source of novel genetic variation as well as be implicated in affecting specific genes which may be linked to important plant traits (as reviewed by Zmieńko et al. 2014). Ferreira de Carvalho et al. (2021) observed copy number variation of one gene implicated in meiosis of resynthesized *B. napus* lines present in two copies in both *B. rapa* and *B. oleracea*. In this study we detected both novel and inherited CNVs in the resynthesized *B. napus* lines analysed, with higher frequencies in homoeologous chromosomes A01-C01, A02-C02, A03-C03, and A09-C09 compared to other chromosomes. The accumulation of CNVs in this region is assumed to be as a result of frequent A-C chromosome pairings. A similar study observed higher frequencies of homoeologous exchanges in the same syntenic regions of chromosomes A1-C1, A2-C2, A3-C3, A9-C8, A9- C9 between the A and the C subgenomes of synthetic *B. napus* (Samans et al. 2017) by using a subset of the same resynthesized lines used in the current study. The presence of novel CNVs in a few domesticated resynthesized lines grown for more than two generations in the present study possibly indicates the inheritance of allelic variants from *B. rapa* and *B. oleracea* parent genotypes which conferred meiotic stability (An et al. 2014). Results from a previous study of allohexaploid hybrids suggested that copy number variation affect gene expression and is closely involved in processes that could affect how regular meiosis takes place in complex interspecific hybrids (Gaebelein et al. 2019).

5.3 The role of inherited allelic variants in meiotic stabilization of *B. napus*

One of the many challenges faced by neopolyploids is the correct chromosome pairing and segregation during meiosis. Although established polyploids rarely have this problem, the molecular basis for the prevention of homoeologous pairing and meiotic stabilization of most polyploid species is still being studied. However, significant progress has been made recently in identifying potential causes of meiotic stability in polyploids (Yant et al. 2013; Morgan et al. 2020, 2022; Higgins et al. 2021). In allopolyploids, the formation of stable bivalents depends on the preferential pairing of chromosomes with the right partner from the same subgenome (Jenczewski and Alix 2004; Lloyd and Bomblies 2016).

Different routes through which allopolyploids achieved meiotic stability have been proposed and researched over the years. Particularly interesting is the hypothesis that pre-existing allelic variants present in the diploid progenitors might be responsible for stabilizing meiosis

(Gonzalo 2022; Katche and Mason 2023). In autotetraploid *A. arenosa*, selection of specific alleles at known meiotic recombination genes seem to achieve improved chromosome segregation, including at least eight meiosis related genes have been implicated to be under selective sweeps related to polyploid adaptation (Yant et al. 2013). Amongst these are alleles of two meiotic chromosome axis genes *ASY1* and *ASY3* as well as their interacting meiotic cohesion subunit *REC8* protein partner implicated in stabilizing meiosis in polyploids via the reduction of multivalent frequency (Morgan et al. 2020, 2022). Therefore, selection of genetic variants at pre-existing loci may have contributed to ensuring regular meiosis in ancient polyploids (Lloyd et al. 2014).

In *B. napus* some progress has also been made in identifying possible causes of meiotic stability, although little or no molecular characterization of putative meiosis gene candidates has been done so far, in contrast to in *Arabidopsis* and wheat. Gonzalo et al. (2019) showed that *MSH4* is important in regulating crossover formation between homologues. Although the formation of normal crossovers is independent of *MSH4* gene duplicate loss, the prevention of homoeologous crossovers was shown to be dosage sensitive for *MSH4* copies (Gonzalo et al. 2019). This is particularly important because most meiotic recombination genes have been shown to rapidly return to single copy (Lloyd et al. 2014), possibly to avoid meiotic irregularities caused by the retention of many gene copies following polyploidy events. However, not all meiosis genes have been reduced to single copy in mesopolyploid species like *B. rapa* and *B. oleracea*, as no obvious change in gene copy number was observed in the subgenomes of *B. rapa* and *B. oleracea* since the formation of *B. napus* (Higgins et al. 2021). Interestingly, a balanced maintenance of meiosis gene copies has been observed in progenitor *B. rapa* and *B. oleracea* (Higgins et al. 2021).

Here, 13 putative meiosis candidate genes were identified which show significant CNV association and presence of putatively harmful mutations in meiosis gene haplotypes (Chapter 4). Of these, three are most interesting because they contain stop codons and splice variants in at least one copy: *RPA1C, MSH2*, and *RECQ4B*. These genes have been shown to have putative meiosis-related gene function in DNA or double strand-break repair, effects on meiotic crossover, and suppression of homoeologous recombination in *Arabidopsis* and other polyploids. Higgins et al. (2021) identified *RPA1C* within the *BnaA9* QTL region responsible for the suppression of homoeologous recombination. *RPA1C* has been shown to be implicated in double strand break repair early in meiosis in Arabidopsis (Aklilu et al. 2014). In this thesis, two copies of *RPA1C* on chromosomes C02 and C09 from the *B. oleracea* progenitor of

resynthesized *B. oleracea* were significantly associated with CNV and fertility, with presence of a radical mutation in both copies and a stop codon in the C02 copy. The copy of *RPA1C* we found on chromosome C09 was homoeologous to the *BnaA09* QTL region identified by Higgins et al. (2021). All the copies identified in this region are promising candidates for future validation. We also identified 3 gene copies of MSH2 in the *B. oleracea* parent genome: two copies on chromosome C06 and one copy on chromosome C03. The presence of a stop codon and a splice variant in one allelic variant of *MSH2* gene was observed on chromosome C06 while the other copy show no significant association with CNV or fertility traits. The copy on chromosome C03 contained a missense codon and was significantly associated with CNV and total seed set. The *MSH2* protein has been shown to regulate meiotic recombination during Prophase 1 (Emmanuel et al. 2006). In Brassica allohexaploids produced from a cross (*B. napus* × *B. carinata*) × *B. juncea, MSH2* was found in the QTL region underlying fertility on chromosome C3 (Gaebelein et al. 2019). *RECQ4A* and *RECQ4B* are paralogs of the *RECQ* genes which have been implicated in limiting crossovers in Arabidopsis (Fernandes et al. 2018; Serra et al. 2018), possibly functioning in the reduction of non-homologous recombination frequency. *AtRECQ4B*, unlike other known eukaryotic *RECQ* homologs, is specifically required to promote but not limit crossover formation (Hartung et al. 2007). Here, *RECQ4B* was identified on chromosome C09 in the *B. oleracea* parent genotype and was significantly associated with CNVs and contained radical SNP mutations as well as the presence of a stop codon gain variant and a splice acceptor. Two copies of *RECQ4A*, which is the other paralog of *RECQ4B*, were found on C08, both of which were significantly associated with CNVs and self-pollinated seeds, with radical SNP mutations as observed by the presence of non-conservative missense codons.

In this thesis, no interesting putative gene candidates from the *B. rapa* parent genotype were observed. This is probably due to the small number of parent genotypes analysed in this study. This is also not so surprising since *B. oleracea* has more than twice the number of unique gene families compared to those present in *B. rapa* (Parkin et al. 2014). Fertility and genome stability in sexually producing organisms is ensured by meiosis, and meiosis genes play a key role in this process. Genetic variation in meiosis genes may have huge effects on genome stability in many plant lineages (Addo Nyarko and Mason 2022). Gene fractionation is a common feature of ancient polyploids (Cheng et al. 2018) which involves loss of one of the copies of a newly duplicated gene, including meiosis genes. It is most likely that *B. napus*, which is only a young species less than 10,000 years old, has undergone little or no major gene fractionation after

whole genome duplication, also with respect to retained meiosis gene copies (Katche et al. 2023). The identification of putative candidate genes from the *B. oleracea* parent genotypes used to produce the resynthesized *B. napus* lines used in this study suggests that selection of meiotic allelic variants including loss of function mutations inherited from diploid progenitors may contribute to a meiotically stable resynthesized *B. napus*. The putative gene candidates identified in this thesis would be relevant for future gene validation either through knock out mutants to observe the effect on plant phenotype or for functional characterization of the genes to investigate the roles they play in the meiosis of *B. napus.*

5.4 Limitations and unanswered questions

This study on recreating genomically stable rapeseed which investigated the fertility and genome stability of a genetically diverse set of resynthesized *B. napus* lines may present some potential limitations. One of its potential limitations is the low number of S_1 resynthesized *B.* napus genotype combinations whose allelic state was predicted from the parent combination used to produce the interspecific cross as well as the low number of resequenced *B. rapa* and *B.* oleracea parent accessions from which the list of meiosis gene candidates was pulled out (Chapter 4). In addition, not all the *B. oleracea* parent genotypes which was used to produce the interspecific *B. napus* lines could be sequenced due to failure of seed germination. No interesting meiosis gene candidates which significantly associated with CNV could be identified from the *B. rapa* parent genotypes based on my analysis because of the small number of genotypes used in this study.

On the other hand, despite the small number of genotypes used in this study, fertility and genome stability as measured by the number of CNVs were still genotype dependent and some interesting meiosis gene candidates were also identified from *B. oleracea*. Therefore, the small number of genotypes used in the second study does not undermine the scientific contributions of this thesis. In the first study (Chapter 3) I screened a large collection of S_1 and later generation resynthesized *B. napus*. However, a large percentage of the lines (66%) were contaminated and/or heterozygous as a result of outcrossing to unknown parents resulting in the assessment of genome stability in only a few pure resynthesized *B. napus* genotypes. Hence, only a small number of putatively stable lines could be identified as indicated by the absence of novel CNVs. It would be worthwhile to investigate meiotic stability of these putatively stable

lines by using molecular cytogenetics methods to observe their chromosome pairing behavior. This would help to validate genome stability of the lines.

Another limitation of this thesis was the presence of unexpected large differences in A genome allele inheritance between progeny sets of resynthesized lines of two *B. rapa* A genome parents; A6 and A7 genotypes. This indicated that these two genotypes as well as all the resynthesized *B. napus* genotype combinations produced by crossing A6 and A7 as A genome parents were heterozygous based on both SNP data analysis as well as the resequencing data. Hence, these two heterozygous A genome parents as well as their progenies were omitted from the meiosis genes analysis. One option would have been to screen both parent genotypes and resynthesized lines for homozygosity to show that there are no allelic differences between progeny sets of the same A or C genome parents before they are considered as experimental materials. Notwithstanding, this could by itself could be considered as an interesting result of the study. The third limitation of this thesis was the absence of both SNP information and resequenced data of the exact *B. rapa* and *B. oleracea* parent individual used to generate the interspecific *B. napus* hybrids. This would have provided the opportunity to screen for allelic variation between the parents and the progenies which would have led to strong conclusions on inheritance of specific allelic variants in the resynthesized lines which might have been responsible for genome stability. However, using the SNP data information of the resynthesized lines as well as the sequence information of the parent genotypes that were available, some interesting putative meiosis genes candidate was still identified for future functional validation.

The fourth limitation of this study is related to the copy number pipeline developed which was used to assess copy number variation in the second study of this thesis (Chapter 4) which has been extensively discussed in the paper. The copy number pipeline used could not efficiently discriminate duplications from other copies greater than two copies, leading to the use of the term “higher copy number” in the study to describe both three or four copies of a chromosomal region. Additionally, the pipeline may also be overestimating both reduced and higher copy number CNV calls. However, the detection of CNV using both hybridization-based SNP array and high-throughput next generation sequencing approaches has been reported to have different limitations (Zmieńko et al. 2014). Mason et al. (2017) reported that failed SNP calls from the 60K *Brassic*a SNP chip reduced the average copy number across certain regions. However, notwithstanding the above limitation, a manual scoring of CNVs across regions of chromosomes of all S_1 resynthesized *B. napus* lines did not detect large differences in CNV calls compared to the CNV pipeline used.

5.5 Conclusions and future perspective

Resynthesized *Brassica* napus provides useful genetic resources that can be utilize in rapeseed breeding for broadening the genetic variation of elite *B. napus* cultivars as well as for understanding the evolutionary question of how two genomes come together to form a species. However, like most newly synthesized polyploids, resynthesized *B. napus* produced via interspecific crosses between *B. rapa* and *B. oleracea* diploid progenitors are often meiotically unstable and infertile, unlike established *B. napus* cultivars. Several pathways to meiotic stabilization in newly formed polyploids have been proposed, one of which is the inheritance of specific allelic variants from diploid progenitor which conferred meiotic stability in newly established polyploids. This thesis tested the hypothesis that allelic variants inherited from diploid *B. rapa* and *B. oleracea* progenitors contributed to meiotic stabilization of *B. napus*. It also aimed to screen a large diverse set of resynthesized *B. napus* genotypes in order to identify fertile and genomically stable lines.

Firstly, the fertility and genome stability of both S1 and later generation resynthesized *B. napus* lines was assessed using the *Brassica* SNP genotyping array. I observed that self-pollinated seed set and genome stability (as measured by the number of CNVs) of S_1 resynthesized *B. napus* produced by crosses between eight *B. rapa* and eight *B. oleracea* lines were significantly affected by the interactions between both diploid parental genotypes.

Fertility and genome stability in all resynthesized *B. napus* lines analysed including S_1 and later generations were genotype dependent. Most of the resynthesized lines had high number of CNVs which associated significantly with reduced fertility. Although most of the lines were shown to be genomically unstable (high number of CNVs), eight putatively stable lines were observed as indicated by the low number of CNVs as well as the absence of novel CNVs.

In the second study, I resequenced eight *B. rapa* and five *B. oleracea* parent genotypes and analyzed nineteen resynthesized *B. napus* lines for allelic variation in a list of meiosis gene homologs. Thirteen putative meiosis gene candidates were observed which were significantly associated with frequency of copy number variants and which contained putatively harmful mutation in meiosis gene haplotypes. This result supports the idea that selection of meiotic allelic variants contributed to meiotic stability of established *B. napus* and would be useful in broadening our understanding of how polyploid species achieved meiotic stability. This

information would also be useful to breeders aiming to use resynthesized lines as direct breeding materials or for the introgression of desirable traits as well as to expand the limited genetic diversity of rapeseed cultivars.

Resynthesized *B. napus* is an indispensable crop improvement tool with the potential to generate new genetic diversity in established rapeseed cultivars. The identification of putatively stable resynthesized lines in this thesis shows us that it is possible to generate genomically stable resynthesized *B. napus* lines. However, this might depend on the parental *B. rapa* and *B. oleracea* genotype cross combination selected for the interspecific crosses. Further research to validate the putatively stable lines identified in this thesis by using both traditional and molecular cytogenetics methods to investigate the chromosome pairing behavior of these lines would be necessary.

Understanding the molecular basis of polyploid adaptations to meiotic challenges remains a hot topic in many studies on polyploid evolution and meiosis. Notwithstanding, there is growing evidence suggesting that specific meiosis genes alleles inherited from diploid progenitors contributed to meiotic stabilization in polyploids (Yant et al. 2013; Morgan et al. 2020, 2022). The meiosis candidate genes identified in this thesis are excellent candidates for future functional validation to characterize knock out mutants using genetic transformation in order to check the effect of the genes on plant phenotype. The validation of the meiosis gene candidates and the understanding of their mechanisms as well as that of other gene regulatory networks which might be involved in meiosis will contribute to our understanding of how meiotic stabilization was achieved in *B. napus* and other polyploids.

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7.0 Appendices

7.1 Appendix 1: Supplementary Information Chapter 3

The Crop Journal Supporting Information

Article title: Fertility, genome stability, and homozygosity, in a diverse set of resynthesized rapeseed lines

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Table S1- Pairwise comparison (TukeyHSD) in total number of self-pollinated seeds between different resynthesized *B. napus* genotypes produced.

Genotypes	p adjusted value
INL2 -CRY1	0.0253759
R76 -CRY1	0.0198807
S2 -CRY1	0.0236401
R76 -FS94	0.0423806
S2 -FS94	0.0497361
INL2 -G2	0.0297092
R76 -G2	0.0233615
S2 -G2	0.0277072
J32-J45 - G39b	0.0257625
INL2 -G56	0.0298818
R76 -G56	0.0235004
S2 -G56	0.0278693
R76 -H113	0.0433345
INL2 -H200	0.0496004
R76 -H200	0.039518
S2 -H200	0.0464385
INL2 -H287a	0.0332404
R76 -H287a	0.0262093
S2 -H287a	0.0310255

INL2 -H327	0.0160728
R76 -H327	0.0124685
S2 -H327	0.0149296
R76 -H365	0.0429746
INL2 -H61	0.0182683
R76 -H61	0.0142095
S2 -H61	0.0169823
INL2 -HIY1	0.0397405
R76 -HIY1	0.0314757
S2-HIY1	0.037142
J151 -INL2	0.010453
J166-INL2	0.0025427
J32-J45 -INL2	0.0003454
J401 -INL2	0.0285261
K138 -INL2	0.0248581
K142-INL2	0.0129851
K199 -INL2	0.0331454
K241-INL2	0.002919
MAY-INL2	0.0120463
MOY5-1 -INL2	0.0323009
OLY2-1 -INL2	0.011674
R53 -INL2	0.0466951
RS10 -INL2	0.0438185
S27 -INL2	0.0171384
VIL1 -INL2	0.0133959
VIY1 -INL2	0.0195247
R76 -J151	0.0080411
S2 -J151	0.0096853
J166-J154	0.033754
J32-J45 -J154	0.0076205
K241-J154	0.0378249
R76 -J166	0.0018738
S2 -J166	0.0023265

S3 -J166	0.0381112
S30 -J166	0.0185007
R76 - J32-J45	0.0002413
S2 - J32-J45	0.0003112
S3 - J32-J45	0.008844
S30 - J32-J45	0.0036682
R76 -J401	0.0224096
S2 -J401	0.0265962
R76 -K138	0.0194659
S2 -K138	0.0231545
R76 -K142	0.0100304
S2 -K142	0.0120463
R76 -K199	0.0261325
S2 -K199	0.0309361
R76 -K241	0.0021557
S2 -K241	0.0026725
S3 -K241	0.0426423
S30 -K241	0.0208757
R76 -MAY	0.0092917
S2 -MAY	0.0111705
R76 -MOY5-1	0.0254507
S2 -MOY5-1	0.0301423
R76 -OLY2-1	0.0089991
S2 -OLY2-1	0.0108233
R76 -R53	0.037142
S2 -R53	0.0436971
RS10 -R76	0.0347945
S27 -R76	0.0133128
S45 -R76	0.0450495
VIL1 -R76	0.010354
VIY1 -R76	0.0152082
S2 -RS10	0.0409845

S27 -S2	0.0159256
VIL1 -S2	0.0124296

Table S2- Pairwise comparison (TukeyHSD) in seeds per ten pods between different resynthesized *B. napus* genotypes produced.

Genotypes	p adjusted value
J32-J45 - G35-J134	0.0069384
K241- G35-J134	0.0300693
S2 - H123	0.027132
R76 -H327	0.0330888
S2 -H327	0.0146281
R76 - H355-S13	0.0397411
S2 - H355-S13	0.0144474
J32- J45 -H94	0.0157703
R76 - HIY1	0.004175
S2 -HIY1	0.001462
J32-J45 -INL2	0.0296841
R76 -INY2	0.0077679
S2 -INY2	0.0028123
R76 -INY3	0.0218796
S2 -INY3	0.0084727
R76 -J166	0.0134057
S2 -J166	0.0055824
R76 -J32-J45	0.0002633
S2 -J32-J45	0.0000762
R76 -J401	0.0497117
S2 -J401	0.022701
R76 -J408	0.0129114
S2 -J408	0.0053648
R76 -K138	0.0129114
S2 -K138	0.0053648
R76 -K147	0.0166699
S2 -K147	0.0063314
R76 -K241	0.0018221
S2 -K241	0.0006139

S2 -K242	0.0291137
R76 -MAY	0.0139175
S2 -MAY	0.0058084
S2 -MOY5-1	0.0235306
S2 -OLL1d	0.034651
R76 -OLL1g	0.0157758
S2 -OLL1g	0.0059694
R76 -OLL1h	0.0463953
S2 -OLL1h	0.0191127
S2 -R140	0.0463953
RS13c -R76	0.0129114
S237 -R76	0.0497117
S39 -R76	0.0212986
VIL1 -R76	0.0480881
S2 -RS13c	0.0053648
S237 -S2	0.022701
S39 -S2	0.0082314
VIL1 -S2	0.0218981
VIY1 -S2	0.0252731

Table 3- Total number of self-pollinated seeds significantly associates with number of CNV in resynthesized *B. napus* lines (Pearson's chi-squared test p < 0.05)

Self-pollinated seeds	high CNV	low CNV
fertile	0	5
highly fertile	0	5
moderately fertile	3	1
poorly fertile	6	2

Table 4- Total number of self-pollinated seeds significantly associated with number of CNVs in resynthesized *B. napus* lines (Pearson's chi-squared test p < 0.05)

Seeds per ten pods	high CNV	low CNV

fertile	0	3
highly fertile	0	5
moderately fertile	3	3
poorly fertile	5	1

Table 5- Percent pollen viability significantly associated with number of CNVs in resynthesized *B. napus* lines (Pearson's chi-squared test $p < 0.05$)

pollen viability	high CNV	low CNV
fertile	**0**	**4**
highly fertile	**0**	**5**
moderately fertile	**4**	**2**
poorly fertile	**6**	**1**

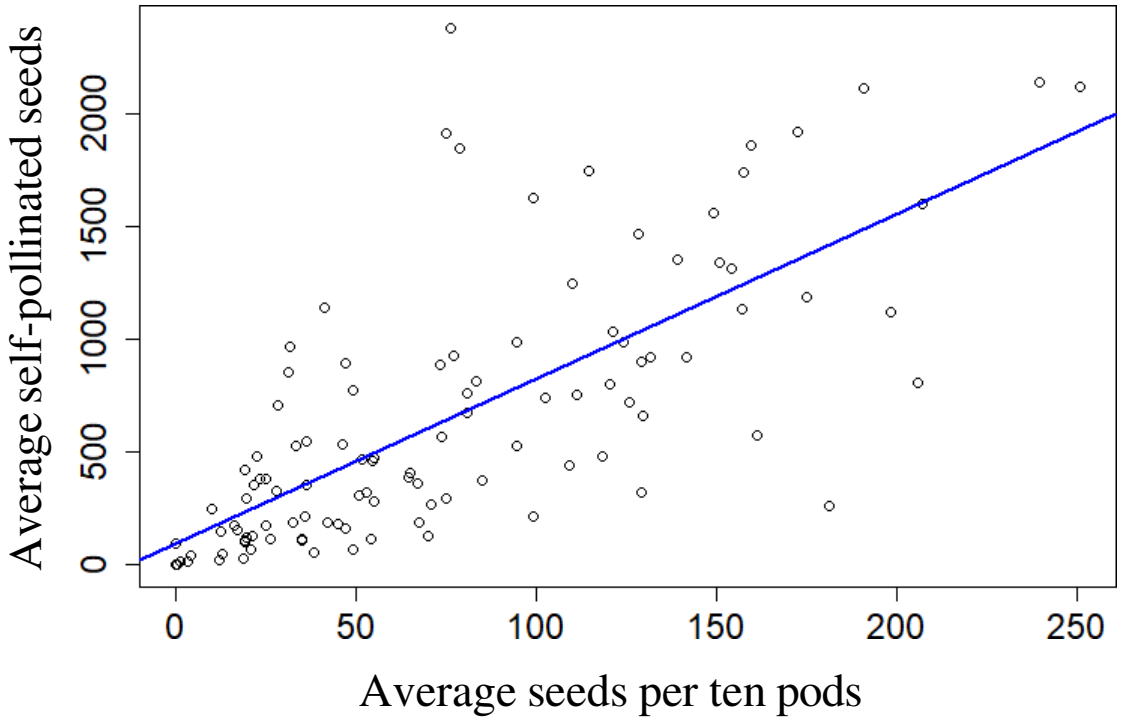

Figure S1- Moderate positive correlation between average number of seeds per ten pods and average self-pollinated seeds (r= 0.72).

7.2 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.

Bonn, 2023 **Elizabeth Ihien**

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