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

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Abstract

Key message Two putative meiosis candidate genes *Bol.MCM8.C05* and *Bol.HEI10.C06* significantly associated with meiosis and/or fertility as well as genome stability were identified in resynthesized *B. napus* lines by Illumina resequencing.

Abstract The genetic diversity in *Brassica napus* (canola, rapeseed) is limited because of few hybridization events and selection for important oil quality traits. One possible means of overcoming this challenge is through resynthesizing *B. napus* hybrids by crossing the diploid parents in order to broaden its gene pool. However, resynthesized *B. napus* lines are often unstable and infertile, unlike *B. napus* cultivars. Meiotic stability in established *B. napus* may have arisen through allele inheritance from the progenitor species. We tested this hypothesis by characterizing 41 resynthesized *B. napus* lines produced by crosses between eight *B. rapa* and eight *B. oleracea* lines for copy number variation resulting from non-homologous recombination events, allele inheritance, and fertility. We resequenced eight *B. rapa* and five *B. oleracea* parent accessions, and analyzed the allelic variation in a list of meiosis gene homologs. SNP genotyping was performed using the Illumina Infinium *Brassica* 60K array for three 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 two putative meiosis gene candidates (*HEI10* and *MCM8*) which were significantly associated with meiosis and/or fertility as well as genome stability. 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 two 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 two different species, known as allopolyploidy (Otto 2007). Polyploidy confers a number of evolutionary advantages (Soltis and Soltis 2000), and many crop species are allo- or autopolyploids.

The *Brassica* genus is one 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 six 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 *B. oleracea* (Mediterranean cabbage, genome C_oC_o) and *B. rapa* (Asian cabbage or turnip, genome A_rA_r) in the last 7500 years, and is thought to be polyphyletic in origin (Allender and King 2010; Chalhoub et al. 2014).

Established *B. napus* cultivars have limited genetic diversity as a result of few hybridization events between the diploid *B. rapa* and *B. oleracea* parents as well as strong breeding selection for zero seed erucic acid and low glucosinolate content (Delourme et al. 2013). Therefore, broadening its genetic diversity is necessary for sustainable rapeseed breeding (Hu et al. 2019). Different approaches have been studied in order to extend genetic variation in the current *B. napus* breeding pool (Seyis et al. 2003; Abel et al. 2005; Girke et al. 2012a; Jesske et al. 2013; Wu et al. 2015; Szała et al. 2019; Nikzad et al. 2020). Several of these studies exploited the use of rapeseed's diploid progenitor species *B. rapa* and *B. oleracea*, which have been shown to possess large genetic variation in both wild and cultivated forms (Mithen et al. 1995; Lazaro and Aguinalalde 1998; Christensen et al. 2011; Aissiou et al. 2018; Maggioni et al. 2020), to produce resynthesized *B. napus*. Therefore, resynthesized lines are useful resources for expanding the genetic diversity in elite *B. napus* cultivars (Becker et al. 1995). Resynthesized lines have been produced by several authors in order to evaluate heterosis and/or agronomic potentials for its introgression into current rapeseed breeding pool (Abel et al. 2005; Seyis et al. 2006; Zhang and Zhou 2006; Zhao et al. 2009; Girke et al. 2012b; Jesske et al. 2013). However, resynthesized lines produced by crossing *B. rapa* and *B. oleracea* have exhibited many undesirable agronomic traits such as low yield and oilseed content, low quality of seeds,

poor winter hardiness, high erucic acid in oil, and high glucosinolate content (Seyis et al. 2003; Zhao et al. 2009; Jesske et al. 2013; Karim et al. 2014). A few studies have reported the use of semi-resynthesized lines obtained by crosses between resynthesized *B. napus* lines and 00-quality *B. napus* cultivar to improve yield and/or oil quality in resynthesized rapeseed (Udall et al. 2004; Seyis et al. 2006; Zhao et al. 2009; Szała et al. 2016, 2019). One study also suggested the production of resynthesized *B. napus* lines via interspecific crosses with 0 or 00-quality *B. rapa* genotypes as a way to improve oil seed content so that resynthesized lines can be used for the improvement of quality and yield in rapeseed breeding (Seyis et al. 2003). Although (Girke et al. 2012a) reported higher yields in some resynthesized genotypes compared to *B. napus* cultivars, low yields still largely remain a major challenge. This may imply that some resynthesized genotypes are more fertile than others and some genetic factors may be responsible for these differences. Hence, a few studies have proposed that genome stability of resynthesized lines must be confirmed before they can be adapted in commercial hybrid breeding programs (Girke et al 2012; Jesske et al 2013).

The question then is why the established *B. napus* species is stable and the resynthesized are unstable. One hypothesis is that *B. napus* may have gained genetic control via the inheritance of specific alleles from its diploid progenitor species (Mason et al. 2015a). A few studies have been conducted to explain genome instability in resynthesized *Brassica napus* allotetraploids (Gaeta et al. 2007; Szadkowski et al. 2010; Xiong et al. 2010), and recently several quantitative trait loci were identified to be present in natural *Brassica napus* that confer reduced homoeologous recombination rates (Higgins et al. 2020). 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*) and Chinese Kale (*B. oleracea* var. *alboglabra*), A- genome genotypes are yellow sarson (*B. rapa* ssp. *trilocularis*), oilseed turnip (*B. rapa* ssp. *oleifera*), and Chinese cabbage (*B. rapa* ssp. *pekinensis*). Abel et al. (2005) produced seeds from 336 cross-combinations between twenty-one *B. rapa* (maternal parent) and sixteen *B. oleracea* lines including a core set of 64 cross-combinations between eight *B. rapa* and eight *B. oleracea* lines. Seeds from 41 resynthesized *B. napus* genotypes produced via embryo rescue, chromosome doubling and self-pollination (S1 generation), as well as 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 semi-winter 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, three seeds from each of 41 synthetic 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* and seven *B. oleracea* parent lines used to produce the resynthesized *B. napus* lines were likewise sown. All seeds germinated, and three plant replicates from each genotype were isolated in bags at flowering to ensure self-pollination.

Fertility assessment in resynthesized *B. napus* lines

Three parameters were scored to describe fertility in resynthesized lines: pollen viability, total number of seeds produced per plant, and number of seeds produced per ten pods. Pollen viability was assessed for two 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 non-viable. The total number of self-pollinated seeds produced per plant and the number of seeds produced per ten pods were counted for each plant after harvesting.

DNA extraction, sequencing and sequence analysis of parental lines

DNA was extracted from young leaf samples using the Doyle and Doyle DNA extraction protocol (Doyle and Doyle 1987). Illumina paired-end sequencing was performed at Novogene Company Limited, United Kingdom on a Illumina HiSeq machine to produce reads of 150 bp length. Quality control was performed using FASTQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), no further processing was found to be necessary. Reads were then mapped onto the respective reference genomes *B. rapa* ‘Chiifu-401-42’ version 2 (Wang et al. 2011) and *B. oleracea* version 2.1 ‘TO1000’ (Parkin et al. 2014) using SOAP2aligner (Li et al. 2009b) using the options –x 100 –m 1000 and otherwise Default. Removal of duplicates, sorting and indexing was carried out with samtools version 0.1 (Li et al. 2009a). 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 S1). SNP annotation was performed using CooVar (Vergara et al. 2012). CNV calling was performed based on read coverage using respective publically available diversity sets for *B. rapa* and *B. oleracea* (Cheng et al. 2016) as control data as described previously (Schiessl et al. 2017).

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

Young leaf samples were collected in 2 ml micro-centrifuge tubes at the 4 to 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/>). 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 was analysed, 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 genome 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 *Brassica oleracea* (CC) genotype controls as well as SNPs which mapped to the C genome but amplified in *Brassica juncea* (AABB) and *Brassica rapa* (AA) genotype controls in > 50% of the controls were filtered out. SNP markers with > 50% heterozygous AB calls in all five *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 $\geq 80\%$ AB calls across individuals. After filtering, 21 938 SNPs were retained: 8369 SNPs in the A genome and 13569 SNPs in the C genome (Supplementary 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. Since the resynthesized lines were produced by chromosome doubling of parent F₁ AC hybrids using colchicine, the allotetraploid hybrids are expected to be homozygous. After quality filtering of SNPs, 100% of the resynthesized *B. napus* genotypes were homozygous as expected.

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

A copy number pipeline was developed in R (Schiessl et al., unpublished). The pipeline uses the logR ratios to make plots for every individual line based on estimated cut off values to score copy number variants. In brief, the pipeline first uses a sliding window of 10 adjacent SNPs to screen if SNPs are below or above a SNP-specific threshold value (threshold SNPs). This threshold value was determined from a diverse *B. napus* population and characterizes the expected distribution of logR ratios. Windows with more than five threshold SNPs were kept and merged in case of physical overlap. The merged regions were re-evaluated for the threshold SNP content, and regions with more than 50% threshold SNPs were retained. Regions were merged if not separated by more than 5 Mb, and regions smaller than 2 Mb were filtered out. Finally, position and type of CNVs were counted and summarized (Fig. 1).

Fig. 1 An example of copy number variant calling in resynthesized *B. napus* lines showing regions of extra copy number (blue), no copies / deletion (red), expected copy (grey), and single / reduced copy (yellow)

Statistical analysis

Genotypic effects on fertility (self-pollinated seeds and seeds per ten pods) and genome stability (as measured by number of CNVs) were tested for due to alleles inherited from either progenitor parents *B. rapa* and *B. oleracea* or the interaction between the two in the resynthesized lines. One-way ANOVA was used to test for significant differences in means followed by Tukey's HSD to test for differences between parent *B. rapa* and *B. oleracea* genotype groups in R v. 3.6.3.

Results

Resynthesized *B. napus* lines show higher fertility than *B. rapa* and *B. oleracea* cultivars as well as comparable fertility to established *Brassica napus* cultivars

Total self-pollinated seeds per single plant in the resynthesized lines ranged from 1 to 2067 (mean 445) (Supplementary Figure S1a, Supplementary File S3), with a mean of 45 seeds per ten pods (range 0 – 148) (Supplementary Figure S1b). Resynthesized lines also showed average pollen viability of 81% (Supplementary Figure S2). Average number of self-pollinated seeds

in resynthesized lines was higher compared to *B. rapa* and *B. oleracea* cultivars (Fig. 2). Average number of seeds per 10 pods and average number of self-pollinated seeds were moderately highly correlated ($r = 0.68$) (Supplementary Figure S3), although there was no significant correlation between pollen viability and either of the seed fertility measures (Supplementary Figures S4a, b).

Fig. 2 Fertility of resynthesized lines measured by the average number of self-pollinated seeds was compared to *B. rapa*, *B. oleracea*, and *B. napus* cultivars

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 ten pod). It was found that the total number of self-pollinated seeds produced was significantly affected by *B. rapa* parent genotype (ANOVA, $p = 0.000483$) (Fig. 3) but not by *B. oleracea* parent genotype (Supplementary Figure S5). Neither *B. rapa* nor *B. oleracea* parent genotype independently affected seeds per ten pods (Supplementary

Figures S6a, b). However, a significant interaction effect (one-way ANOVA, $p = 4.31e-06$) 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 (Supplementary Table S1).

Fig. 3 *B. rapa* maternal genotype significantly affected the total number of self-pollinated seeds produced (ANOVA, $p = 0.000483$, Tukey's HSD, $p < 0.05$) in resynthesized *B. napus* lines produced from different combinations of *B. rapa* crossed with *B. oleracea*

Frequent copy number variation 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. 4). The total number of CNVs detected varied widely between resynthesized *B. napus* lines (Supplementary Figure S7, Supplementary File S3).

Fig. 4 Genome-wide copy number variation was detected in resynthesized *B. napus* lines. Deletions (blue), reduced copy (red), and higher copy number (green)

***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 two parent genotypes was significant, such that there were significant differences between resynthesized lines (ANOVA, $p = 3.15 \times 10^{-6}$; Fig. 5, Supplementary Table S2). Hence, the number of CNVs detected was affected by different cross combinations of *B. rapa* and *B. oleracea* parent genotypes.

Fig. 5 Total number of CNVs varied significantly between resynthesized *Brassica napus* (rapeseed) lines from different genotype combinations (ANOVA, $p = 3.15 \times 10^{-6}$ Tukey's HSD, $p < 0.05$). 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 AXCX

The relationship between genome stability (as measured by copy number variation) and fertility was assessed in the resynthesized *B. napus* lines. Genome stability was negatively correlated with fertility ($r = -0.22$) in resynthesized *B. napus* lines (Supplementary Figure S8). This relatively weak relationship indicates that other factors apart from genome stability might also contribute to fertility in resynthesized lines.

Allelic variation in *BolHEI10.C07* and *BolMCM8.C05* is associated with number of CNVs and pollen viability

Eight parent accessions of *B. rapa* (A4, A6, A7, A8, A9, A13, A16, A19) and five parent accessions of *B. oleracea* (C34, C36, C37, C46, C47) were resequenced and the allelic variation analysed in a list of meiosis gene homologs. 3982 SNPs in *B. rapa* meiosis genes were detected, of which 904 were non-synonymous and 2 were splice variants. In *B. oleracea* meiosis genes, 2594 SNPs were detected, of which 729 were non-synonymous, 4 were splice variants and 5 were stop codon gains. Moreover, CNVs in meiosis genes were detected by analysing coverage. It was found that 96 of the 197 *B. rapa* meiosis gene copies carried a deletion in at least one accession, out of which two were deleted in all 8 accessions, and 90 gene copies carried a duplication. In *B. oleracea*, 33 gene copies out of 193 were deleted in at least one accession, one of them in all accessions, and 57 were duplicated.

From these data, the allelic state of the S1 *B. napus* accessions was inferred by combining the respective parents. Using the phenotypic data for all available cross combinations which could be tested in the greenhouse, we classified those combinations into “stable” (7 combinations), “medium” (10 combinations), “unstable” (10 combinations) and “missing/no data” (13 combinations) (Table 1). In the next step, variants or haplotypes which were present in stable lines were identified, while being absent from non-stable lines, and vice versa. Likewise, three gene copies with unaltered or harmless haplotypes in “stable” combinations were found, while five, four and seven combinations in “medium”, “unstable” and “missing” carried a putatively harmful mutation (non-conservative, radical SNP mutation, splice variant or deletion), which were *Bol.MCM8.C05*, *Bol.HEI10.C06* and *Bol.MS5/TDM_copy2.C07*. A further putatively harmful haplotype for *Bol.CUL4.C07* was found in all “stable” combinations, but only in five, six and six combinations in the respective other sets. In 42 other copies, no variation in “stable” combinations and harmful variation in other sets was found, but with low frequency. Those were not considered further, but could constitute interesting candidate genes for minor effects in future.

Table 1 Classification of resynthesized *B. napus* cross combinations into stable, unstable, and intermediate by estimating CNVs in meiosis genes using phenotypic data. Genotypes without data (no plants) are indicated with “-”

	C34 var bot dh	C47 var alb il	C37 var bot dh	C36 var bot dh	C46 var alb il
A6 var olei dh	-	Intermediate	Intermediate	-	Unstable
A9 var olei dh	-	Intermediate	-	Unstable	Intermediate
A7 var olei dh	Intermediate	-	Unstable	Unstable	Stable
A4 var tril il	Unstable	Unstable	Intermediate	Intermediate	Stable
A8 var tri dh	Unstable	Intermediate	Unstable	Stable	Intermediate
A16 var tri il	-	Intermediate	Unstable	-	Stable
A19 var tri il	-	Unstable	Stable	Stable	-
A13 var pek dh	-	-	-	Stable	-

*var olei represents *B. rapa* var. *oleifera*, var tri represents *B. rapa* var. *trilocularis*, var bot represents *B. oleracea* var. *botrytis*, and var alb represents *B. oleracea* var. *albogabra*. *dh means double haploid while il means inbred lines

To check if these variants were associated with meiotic stability and/ or fertility, the respective phenotypes between combinations were compared with different haplotypes. For *Bol.CUL4.C07* and *Bol.MS5/TDM_copy2.C07*, we could not find any statistically significant differences between the haplotypes (data not shown). For both *Bol.MCM8.C05* and *Bol.HEI10.C06*, however, significant differences were found. *Bol.MCM8.C05* originally had three haplotypes: unchanged (Haplo 1), deleted (Haplo 2), or carrying a conservative missense codon (Haplo 3). Further investigation showed that the latter is very likely also a deletion, as coverage was only slightly above threshold, hence haplotypes 2 and 3 were merged. Haplo 1 and 2 of *Bol.MCM8.C05* differed significantly for total number of CNVs and percent pollen viability (Fig. 6a, d), but not for seeds per ten pods and total seed set (Fig. 6b, c). Haplo 1 was only present in C46, while Haplo 2 was present in C36 and C37, and Haplo 3 was present in C34 and C47.

Haplotypes of *Bol.MCM8.C05* gene

Fig. 6 Phenotypic association for haplotypes of *Bol.MCM8.C05* gene in resynthesized *B. napus* lines was measured by a) total number of CNVs b) total seed set c) seeds per ten pods d) percent pollen viability. Asterisks on boxplots represent significant association of haplotypes to phenotypes (pairwise t.test, $p < 0.05$)

Meiotic stability candidate gene *Bol.HEI10.C06* had three haplotypes: unchanged (Haplo 1), carrying two conservative missense mutations (haplo 2) and carrying a radical missense mutation (Haplo 3). Haplo 1 and 2 differed significantly for total number of CNVs, total seed set and pollen viability (Fig. 7a, b, d). Haplo 1 and 3 were significantly different for total number of CNVs, while Haplo 2 and 3 were significantly different for total seed set (Fig. 7b). Haplo 1 was again only present in C46, while Haplo 2 was present in C36 and C37, and Haplo 3 was present in C34 and C47. However, no significant differences for seeds per ten pods were observed across all three haplotypes (Fig. 7c).

Haplotypes of *Bol.HEI10.C05* gene

Fig. 7 Phenotypic association for haplotypes of *Bol.HEI10.C05* gene in resynthesized *B. napus* lines was measured by: a) total number of CNVs b) total seed set c) seeds per ten pods d) percent pollen viability. Asterisks on boxplots represents significant association of haplotypes to phenotypes (pairwise t.test, $p < 0.05$)

Genetic analyses of resynthesized *Brassica napus* lines

Analysis of the genetic background of the parental lines used in this study showed that maternal *B. rapa* var. *oleifera* lines produced less stable resynthesized lines (1 out of 10 considered as stable) than when *B. rapa* var. *trilocularis* was used as a maternal parent (6 out of 17 stable resynthesized lines recovered) (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 analyzed genome stability (as measured by copy number variants) and fertility (self-pollinated seed-set and seeds per ten pods) in resynthesized *Brassica napus* after one generation of self-pollination. 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 rapeseed lines from different genetic backgrounds also vary significantly in both fertility and genome stability. We identified two putative meiosis candidate genes which were significantly associated with meiosis and/or fertility as well as genome stability in our resynthesized *B. napus* lines.

We detected no evidence of heterozygosity as a result of outcrossing in our S1 self-pollinated resynthesized *B. napus* lines, as no large blocks of heterozygosity was present in our marker data: we observed 100% homozygosity in our lines, as expected. Mason et al. (2018) detected heterozygosity in diverse semi-spring type self-pollinated lines grown in the field for several generations, attributed to outcrossing as a result of the presence of large blocks of heterozygous calls across individuals in marker data. Our avoidance of contamination possibly results from the fact that the lines used were grown in the glasshouse under controlled conditions, and have only undergone one round of self pollination as opposed to field trials grown for several generations in previous studies (Cresswell 1994; Pascher et al. 2010; Bayer et al. 2015; Mason et al. 2018). Most lines were also partially fertile, increasing the chance of self-pollination rather than cross-pollination.

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; two meiosis events). This observation is likely due to the selection of specific allelic variants from the parent genotypes which influenced genome stability in the resynthesized lines. Gaeta et al. (2007) suggested that *B. napus* might have initially been unstable, but that alleles responsible for genetic control of meiosis inherited from one or both diploid progenitors may have been selected for over time, possibly by conferring improved seedset. In *Arabidopsis arenosa*, selection of specific alleles of meiosis genes seems to be responsible for reduced crossover frequency, resulting 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 meiosis in polyploid *Arabidopsis arenosa* (Morgan et al. 2020), and introgression of meiosis gene alleles from *A. arenosa* was found to help stabilize tetraploid *A.*

lyrata (Marburger et al. 2019). Similarly, selection of genetic variants at pre-existing loci may have contributed to form stable meiosis in ancient polyploidy *Brassica* (Lloyd et al. 2014).

Our results show a negative correlation between genome stability (CNVs) and fertility (Spearman correlation $r = -0.3$) in resynthesized *B. napus*. Evidence that chromosome rearrangement contributes to phenotypic variation has previously been shown in resynthesized *B. napus* lines (Gaeta et al. 2007; Xiong et al. 2011). However, Mason et al. (2014) found no relationship between losses of chromosome segment and seed set or pollen viability in self-pollinated allohexaploid hybrids. By contrast, Gaebelein et al. (2019) observed that translocations, and especially the loss or retention of chromosomes present only in single copy, showed the greatest influence on fertility (seedset) in mapping populations of synthetic allohexaploids (*B. napus* $\times$ *B. carinata*) $\times$ *B. juncea*. In natural *B. napus*, inheritance of unbalanced translocation events in mapping populations was also associated with a fertility penalty (Osborn et al. 2003). In resynthesized *B. napus* lines, Xiong et al. (2011) showed that the 5% of lines with highest fertility contained the expected parental contribution to the karyotype of *B. napus*. 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 copy number variation was significantly negatively associated with fertility (self-pollinated seedset). Although the detected correlation was low, this indicates that other factors apart from fertility might be contributing to genome stability in resynthesized *B. napus* lines.

Resynthesized lines showed higher fertility than *B. rapa* and *B. oleracea* check cultivars 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. These results support 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. 2005a). However, with additional generations, unstable meiosis may have increasingly severe effects on fertility, as previously observed in homozygous synthetic allohexaploid *Brassica* lines (Mwathi et al. 2019). We observed a wide range of fertility across the resynthesized *B. napus* lines, with seed set in a few lines comparable to that of the natural *B. napus* controls used. 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 resynthesized *B. napus* populations, and found very low fertility compared to natural

B. napus using both fertility measures. Guo et al. (2016) analyzed resynthesized *B. napus* lines for fertility by assessing the number of primary branches as well as number of seeds per silique, and detected lower fertility compared to natural *B. napus* cultivars. 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 produce the resynthesized *B. napus* population. In support of these studies, we also hypothesized that self-incompatibility alleles present in some parent *B. rapa* genotypes used to produce our resynthesized lines might be responsible for low fertility in a few lines (9 genotypes produced <15 seeds).

Two putative meiosis gene candidates *Bol.MCM8.C05* and *Bol.HEI10.C06* which were significantly associated with meiosis and/or fertility as well as genome stability (as measured by number of CNVs) were identified. Seear et al., (2020) used a similar approach, and also associated the presence of diploid haplotypes to meiotic stability by analyzing 52 tetraploid *A. lyrata* and *A. arenosa* plants using high-throughput sequencing of eight meiosis genes. The meiosis gene candidates *MCM8* and *HEI10* identified in our study are well-conserved among species (Chelysheva et al. 2012; Li et al. 2018) and have both been shown to play a role in meiotic recombination in eukaryotes (Blanton et al. 2005; Wang et al. 2012; Crismani et al. 2013; Li et al. 2018; Serra et al. 2018) *HEI10* (*Human Enhancer of Invasion 10*) which is a RING-finger containing protein belonging to a class of E3 ubiquitin ligases, has been observed by a few studies to increase crossover (CO) formation and chiasma frequency in *Arabidopsis* (Chelysheva et al. 2012; Ziolkowski et al. 2017; Serra et al. 2018), rice (Wang et al. 2012; Li et al. 2018), and mice (Ward et al. 2007). *MCM8* (Mini-chromosome maintenance 8) protein is a member of the eight MCM family proteins (*MCM2–9*), that all share a well conserved helicase has been shown to function in efficient meiotic double strand break (DSB) repair but not in CO formation (Lutzmann et al. 2012; Crismani et al. 2013). However, one study in *Drosophila* observed that a mutation in REC, an orthologue of *MCM8*, reduced crossover frequency by 95% in *Drosophila* (Blanton et al. 2005). *MCM8* has been observed to play a role in DNA replication, especially in the maintenance and progression of replication fork (Gozuacik et al. 2003; Maiorano et al. 2005) as well as functioning in the suppression of replication stress in *B. rapa* and *B. oleracea* species (Shanmugam et al. 2017). Serra et al., (2018) suggested that elevated levels of meiotic crossover in *HEI10* and *recq4a/recq4b* may be associated with significantly reduced seed-set in *Arabidopsis*. Other studies in *Arabidopsis thaliana*, rice and using knock-out mice observed poor fertility in *hei10* and *mcm8* mutants as

a result of abnormal meiosis (Ward et al. 2007; Chelysheva et al. 2012; Lutzmann et al. 2012; Crismani et al. 2013; Li et al. 2018), thereby indicating that *MCM8* and *HEI10* play vital roles in ensuring fertility and/or meiosis. These results are in support of our study where the unchanged variant (Haplo 1) was only present in *B. oleracea* parent C46 and contributes to a lower number of CNVs, and higher pollen viability. Hence, the respective allelic variants of *Bol.MCM8.C05* and *Bol.HEI10.C06* are putatively good candidates for the variation in homoeologous recombination frequency observed in our study. However, validation of *HEI10* and *MCM8* still has to be done in order to confirm the roles of these genes in *Brassica* species.

In this study, we detected copy number variation at a high frequency across the A and C genome. We also found fewer deletions (5.6%) than either reduced (one missing copy) or higher copy number (one or two extra copies as predicted by the pipeline) variants. 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 regions (deletion-duplication events) between A and C homeologous chromosomes A1-C1, A2-C2, and A9-C9 in both resynthesized and natural *B. napus* (Chalhoub et al. 2014; Samans et al. 2017; Mason et al. 2018). Chalhoub et al. 2014 identified 17 translocated regions between the A and C genomes by analyzing Darmor *B. napus* cultivars as well as 7 diverse *B. napus* genotypes and detected homeologous exchanges between A and C homeologous chromosomes A1–C1, A2–C2, and A9–C9. In synthetic *B. napus* lines, an average of 23 homeologous exchanges per synthetic line were detected (Samans et al. 2017). Samans et al. 2017 analyzed 52 highly diversified *B. napus* genotypes including 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 natural accessions as well as more areas with deletions than duplications. In contrast to the above mentioned 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* material has undergone only one self-pollination event (two meiosis). Hence, any novel variants which have arisen are unlikely to be “fixed”, as the products of a single homoeologous crossover event during meiosis rarely segregate together: two recombinant chromatids are produced from one 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 three copies of an allele or more copies (4+) were present, leading us to use a combined “higher copy number” category. Our copy number pipeline may also be over-estimating reduced 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 positive error rates (Mason et al. 2017). In addition, lower signal detection 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 NGS approaches used for the detection of CNVs have different limitations (Zmieńko et al. 2014); high-coverage sequencing likely provides a more robust method of calling 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).

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 copy number variation events, but that the presence of such events may not always be detrimental to fertility in resynthetic *B. napus* lines. Resynthesized *B. napus* lines have the potential to increase the genetic variation in the narrow *B. napus* breeding pool, but would be easier to use (e.g. to provide a divergent germplasm pool for hybrid breeding) if they were genomically stable. Our study suggests that meiotic stability in *B. napus* arose via selection of allelic variants from its diploid progenitor species. Identifying alleles for higher genomic stability is therefore of high importance when incorporating new resynthesized *B. napus* lines into adapted oilseed rape breeding programs.

Author Contribution Statement

Resynthesized *B. napus* 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 and ZL analysed sequencing data, and SVS provided final analyses and results related to meiosis gene variation. AM, AS, HB, ZL and SVS contributed to critical revisions of the manuscript.

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Declarations

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

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figure legends

Figure 1 An example of copy number variant calling in resynthesized *B. napus* lines showing regions of extra copy number (blue), no copies / deletion (red), expected copy (grey), and single / reduced copy (yellow)

Figure 2 Fertility of resynthesized lines measured by the average number of self-pollinated seeds was compared to *B. rapa*, *B. oleracea*, and *B. napus* cultivars

Figure 3 *B. rapa* maternal genotype significantly affected the total number of self-pollinated seeds produced (ANOVA, $p = 0.000483$, Tukey's HSD, $p < 0.05$) in resynthesized *B. napus* lines produced from different combinations of *B. rapa* crossed with *B. oleracea*

Figure 4 Genome-wide copy number variation was detected in resynthesized *B. napus* lines. Deletions (blue), reduced copy (red), and higher copy number (green).

Figure 5 Total number of CNVs varied significantly between resynthesized *B. napus* lines from different genotype combinations (ANOVA, $p = 3.15e-06$ Tukey's HSD, $p < 0.05$)

Figure 6 Phenotypic association for haplotypes of *Bol.MCM8.C05* gene in resynthesized *B. napus* lines was measured by: a) total number of CNVs b) total seed set c) seeds per ten pods d) percent pollen viability. Asterisks on boxplots represents significant association of haplotypes to phenotypes

Figure 7 Phenotypic association for haplotypes of *Bol.HEI10.C05* gene in resynthesized *B. napus* lines was measured by: a) total number of CNVs b) total seed set c) seeds per ten pods d) percent pollen viability. Asterisks on boxplots represents significant association of haplotypes to phenotypes

Table legends

Table 1 Classification of resynthesized *B. napus* cross combinations into stable, unstable, and intermediate by estimating CNVs in meiosis genes using phenotypic data. Genotypes without data (no plants) are indicated with “-”

Supplementary information

Supplementary Figures

Figure S1 Fertility of resynthesized *B. napus* lines was measured by the total number of self-pollinated seeds produced, as well as the number of seeds per ten pods a) total number of self-pollinated seeds produced in resynthesized lines and b) number of seeds per ten pods in resynthesized lines

Figure S2 Percent pollen viability across individuals in resynthesized *B. napus* lines

Figure S3 Moderate positive correlation between average number of seeds per ten pods and average self-pollinated seeds ($r = 0.68$)

Figure S4a Correlation between average percent pollen viability and average self-pollinated seed set ($r = 0.06466$)

Figure S4b Correlation between average percent pollen viability and average seeds per ten pods ($r = 0.2207937$)

Figure S5 *B. oleracea* parent genotypes show no significant association with total number of self-pollinated seeds (ANOVA, $p=0.068$) in resynthesized *B. napus* lines

Figure S6a *B. rapa* maternal genotype show no significant effect on the number of seeds per ten pods (ANOVA, $p = 0.658$) in resynthesized *B. napus* lines produced from different combinations of *B. rapa* crossed with *B. oleracea*

Figure S6b *B. oleracea* paternal genotype show no significant effect on the number of seeds per ten pods (ANOVA, $p = 0.0982$) in resynthesized *B. napus* lines produced from different combinations of *B. rapa* crossed with *B. oleracea*

Figure S7 Number of copy number variants present varied widely between different synthetic *B. napus* lines

Figure S8 Negative correlation between average number of self-pollinated seeds and average number of copy number variants in 41 resynthesized *B. napus* lines ($r = -0.2$)

Figure S9 Resynthesized lines produced by chromosome doubling of parent F₁ AC hybrids were completely homozygous after quality filtering

Supplementary Tables

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

Table S2 Pairwise comparisons (Tukey's HSD) in copy number variation between different resynthesized *B. napus* genotypes produced

Supplementary Files

File S1 Meiosis gene positions determined by BLAST

File S2 SNP genotyping data for all S1 resynthesized individual lines across the A and C genomes after quality control

File S3 Phenotype information for all resynthesized S1 lines

Figures

Figure 1

An example of copy number variant calling in resynthesized *B. napus* lines showing regions of extra copy number (blue), no copies / deletion (red), expected copy (grey), and single / reduced copy (yellow)

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Fertility of resynthesized lines measured by the average number of self-pollinated seeds was compared to *B. rapa*, *B. oleracea*, and *B. napus* cultivars

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B. rapa maternal genotype significantly affected the total number of self-pollinated seeds produced (ANOVA, $p = 0.000483$, Tukey's HSD, $p < 0.05$) in resynthesized *B. napus* lines produced from different combinations of *B. rapa* crossed with *B. oleracea*

Figure 4

Genome-wide copy number variation was detected in resynthesized *B. napus* lines. Deletions (blue), reduced copy (red), and higher copy number (green)

Figure 5

Total number of CNVs varied significantly between resynthesized *Brassica napus* (rapeseed) lines from different genotype combinations (ANOVA, $p = 3.15e-06$ Tukey's HSD, $p < 0.05$). 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 AXCX

Figure 6

Phenotypic association for haplotypes of *Bol.MCM8.C05* gene in resynthesized *B. napus* lines was measured by a) total number of CNVs b) total seed set c) seeds per ten pods d) percent pollen viability. Asterisks on boxplots represent significant association of haplotypes to phenotypes (pairwise t.test, $p < 0.05$)

Figure 7

Phenotypic association for haplotypes of *Bol.HEI10.C05* gene in resynthesized *B. napus* lines was measured by: a) total number of CNVs b) total seed set c) seeds per ten pods d) percent pollen viability. Asterisks on boxplots represents significant association of haplotypes to phenotypes (pairwise t.test, $p < 0.05$)

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