Institut für Nutzpflanzenwissenschaften und Ressourcenschutz (INRES)
Fachbereich Planzenzüchtung

Genome stability assessment in new diverse *Brassica* allohexaploid crop types (AABBCC) using molecular karyotyping

Dissertation

zur Erlangung des Grades
Doktorin der Agrarwissenschaften (Dr. agr.)

der Landwirtschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

von

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Bonn, 2024

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Tag der mündlichen Prüfung: 07.05.2024

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn.

Abstract

Interspecific hybridization and polyploidization processes are known to confer advantages, such as hybrid vigor and increased environmental tolerances. Although cultivated diploid and allotetraploid *Brassica* species which contain different combinations of the A, B, and C genomes exist, there is no naturally occurring allohexaploid containing all three genomes (AABBCC). Despite this, there are traits in each of the *Brassica* species, that if combined, can potentially produce a new species with many advantageous features. Although hexaploids can be produced via human intervention, these neo-polyploids have quite unstable genomes and usually suffer from severe genome reshuffling. Whether these genome rearrangements continue in later generations and follow similar, reproducible patterns between different lines, is still unknown. This thesis aims to investigate genomic stability, chromosomes inheritance, seed fertility, and crossability between and within four *Brassica* allohexaploid types (2n = AABBCC = 54): naponigra (*B. napus* × *B. nigra*), carirapa (*B. carinata* × *B. rapa*), junleracea (*B. juncea* × *B. oleracea*), and NCJ (*B. napus* × *B. carinata* × *B. juncea*). Genomic stability was investigated using the Illumina Infinium *Brassica* 90K SNP array genotyping. Karyotype stability varied between genotypes. Although some genomic regions were more likely to be duplicated, deleted or rearranged, a consensus pattern was not shared between genotypes. Significant differences between genotypes and within lineages were found for frequencies of euploids and rearrangements, with one NCJ line showing relatively high karyotype stability. Only 3.2% of allohexaploid plants investigated were euploids. Hybridization between different allohexaploids was mostly achievable, with 0 - 4.6 seeds per flower bud on average, and strong maternal genotype effects were also found. Novel F_1 hybrids between allohexaploid lineages showed similar fertility and stability to their parents. Meiosis analysis of the new F_1 hybrids showed the production of on average 8.6 new rearrangements, with no improvement in genome stability, despite increased heterozygosity. According to the findings in this thesis, synthetic *Brassica* allohexaploids can develop genomic stability in a few generations, but this occurs at very low frequencies and may not always be under selective pressure, due to the unforeseeable link between fertility and karyotype constitution in these hybrid types.

Publications

The following Chapters of this thesis have been prepared for journal submission:

Chapter 2: "Using wild relatives and related species to build climate resilience in *Brassica* crops"

Authors: Daniela Quezada-Martinez, Charles P. Addo Nyarko, Sarah V. Schiessl, Annaliese S. Mason.

Published in Theoretical and Applied Genetics (2021) 134 (3), DOI: 10.1007/s00122-021-03793-3

My contribution to this publication: Writing the "Useful traits identified in *Brassica* crops and wild allies" section and critical revision of the manuscript.

Chapter 3: "Allele segregation analysis of F_1 hybrids between independent *Brassica* allohexaploid lineages"

Authors: Daniela Quezada-Martinez, Jun Zou, Wenshan Zhang, Jinling Meng, Jacqueline Batley, Annaliese S. Mason

Published in Chromosoma (2022) 131: 147-161, DOI: 10.1007/s00412-022-00774-3

My contribution to this publication: carried out the experimental analyses, prepared the results figures and tables, and drafted the manuscript.

Chapter 4: "Karyotype instability varies by species and genotype combination in allohexaploid *Brassica"*

Authors: Daniela Quezada-Martinez, Jacqueline Batley, Annaliese S. Mason

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My contribution to this publication: conceptualized the experiment together with ASM, generated the plant material, managed the crossings, self-pollination, harvesting, DNA extraction, carried out the experimental analyses, prepared the results, figures, and tables, and drafted the manuscript.

Contents

1. LITERATURE REVIEW 1

1.1 Hybrids and Polyploids 1
1.1.1 Polyploids in agriculture 4
1.1.2 Problems with recent polyploids 6
1.1.3 Copy number variation in polyploids 9

1.2 *Brassica* genus 12
1.2.1 Economic importance of *Brassica* species 16
1.2.2 Crossability between *Brassica* species 18
1.2.3 Meiosis in *Brassica* 21
1.2.4 Making new polyploid crop types in *Brassica* 22

1.3 *Brassica* allohexaploids 24

2. USING WILD RELATIVES AND RELATED SPECIES TO BUILD CLIMATE RESILIENCE IN *BRASSICA* CROPS. 29

2.1 Abstract 30

2.2 Climate change will result in a higher frequency of extreme weather events and increased pest and disease loads 31

2.3 The use of wild relatives and related species for crop improvement in *Brassica* 33

2.4 Useful traits identified in *Brassica* crops and wild allies 35
2.4.1 Insect resistance traits 35
2.4.2 Disease resistance traits 38
2.4.3 Abiotic stress tolerances 40
2.4.4 Other traits of agronomic interest 42

2.5 Hybridization for crop improvement in *Brassica* 43

2.6 Transferring useful traits from wild relatives to crop species: how does it work? 45

2.7 Improving our chances of recovering introgressions of useful traits from relatives into crops to build climate resilience 48

2.8 Conclusions 52

3. ALLELE SEGREGATION ANALYSIS OF F_1 HYBRIDS BETWEEN INDEPENDENT *BRASSICA* ALLOHEXAPLOID LINEAGES. 53

3.1 Abstract 54

3.2 Introduction 55

3.3 Materials and methods 59
3.3.1 Plant material and crossing scheme 59
3.3.2 DNA extraction and SNP analysis 60
3.3.3 Chromosome count and molecular karyotyping 61
3.3.4 Allele segregation in the F1 hybrid 63

3.4 Results 63
3.4.1 Pre-existing fixed rearrangements in *Brassica* parental genotypes 63
3.4.2 Chromosome inheritance and fixed karyotype changes in NCJ and carirapa allohexaploid types 64
3.4.3 Crossing NCJ and carirapa allohexaploids: F1 hybrid and allele segregation 66
3.4.4 Allele segregating per population: 67

3.5 De novo rearrangements and inheritance in the F1 hybrids 73

3.6 Discussion 75

3.7 Conclusions 80

4. KARYOTYPE INSTABILITY VARIES BY SPECIES AND GENOTYPE COMBINATION IN ALLOHEXAPLOID *BRASSICA* 81

4.1 Abstract 82

4.2 Introduction 83

4.3 Materials and methods 85
4.3.1 Plant material: 85
4.3.2 Genome stability analysis: 86
4.3.3 Crossings between four allohexaploid types: 87
4.3.4 F_1 hybrids and parents: 87
4.3.5 Plant growth conditions, fertility, and plant data collection: 87
4.3.6 Crossing success: 88
4.3.7 DNA extraction and SNP genotyping: 89
4.3.8 Copy number variant analysis: 90
4.3.9 Statistical analysis: 91

4.4 Results 92
4.4.1 Unexpected genome instability in *Brassica* parental species 92
4.4.2 Chromosome number in *Brassica* hexaploids 93
4.4.3 Chromosome number variants in the NCJ allohexaploid type 94
4.4.4 Chromosome number variants in the carirapa allohexaploid types 96
4.4.5 CNV and karyotype rearrangements in *Brassica* hexaploids 98
4.4.6 Effects of genotype and allohexaploid type on total CNVs 104
4.4.7 Translocation events and subgenome bias in *Brassica* allohexaploids: fixed duplication/deletion events 105

4.4.8 Translocation events and subgenome bias in *Brassica* allohexaploids: segregating events (single copy / extra copy translocations) 107
4.4.9 Crossing *Brassica* allohexaploid genotypes 108
4.4.10 F_1 hybrids compared to the parents: 111
4.4.11 CNVs and seed set in parents and F_1 progeny 112

4.5 Discussion 114

4.6 Conclusions 121

5. GENERAL DISCUSSION 122

6. FUTURE WORK 130

7. CONCLUSION 132

8. APPENDIX 133

8.1 Supplementary Figures and Tables 133

9. REFERENCES 142

1. Literature review

1.1 Hybrids and Polyploids

Hybridization between (intraspecific) and within (interspecific) species is a common occurrence in vascular plants (Whitney *et al.*, 2010) playing an important role in evolution (Arnold, 1992). In general, hybridization can lead to for example, production of a new species, exchange of genetic material, the new hybrid may occupy novel environments or become a trap for pests (Arnold, 1992), and the best known effect of hybrid vigor (Groszmann *et al.*, 2013). Hybridization can be also accompanied by chromosome doubling, giving origin to a polyploid organism. Polyploids refers to an organism or cell that contains two or more sets of chromosomes (Chen 2010). Polyploids can be categorized as autopolyploids, where the new chromosome sets originated from the same individual or from within a species (intraspecific hybridization), or as allopolyploid, originating from the hybridization of two different species (interspecific hybridization) (Otto, 2007).

Polyploidization, or whole-genome duplication events (WGD), have occurred in all flowering and seed plants (Bowers *et al.*, 2003; Jiao *et al.*, 2011). To date, 49 WGD events have been identified in plants, with 38 corresponding to tetraploidization and six to hexaploidization (Cheng *et al.*, 2018). Polyploids can also be categorized based on when they are thought to have originated. This can be done by adding a prefix neo- (< 5 million years ago), meso- (5 - 30 Mya), and paleo- (> 30 Mya), with the special case of "recent" for those newly synthesized (Cheng *et al.*, 2018).

One of the immediate effects of polyploidization is the increment in cell size, growth rate and organ size (known as "gigas" effect) (Otto & Whitton, 2000). Some other parts of the plant such as pollen and stomata also increase their size and are hence, often used as a marker of ploidy (Otto & Whitton, 2000). Surprisingly, even though cell size might increase, this does not necessarily correlate in all cases with an increase in adult plant size (Otto, 2007).

As a result of polyploidy, the newly formed organism has advantages such as gene redundancy (Comai, 2005) that can help to mask deleterious recessive alleles producing functional diversification of redundant gene copies (Sattler *et al.*, 2016) and progressive heterosis in allopolyploids and heterozygous autopolyploids (Birchler *et al.*, 2010). Further effects of

polyploidy can include rapid genomic change (Song *et al.*, 1995), epigenetic gene silencing (Comai *et al.*, 2000), DNA methylation (Madlung *et al.*, 2002), post-polyploidization activation of transposable elements that were inactive in the parental species (Wendel, 2000), and gene loss (Kashkush *et al.*, 2002).

There are two very important stages in the life of polyploids: formation and establishment (Ramsey & Schemske, 1998). Several factors can influence the ratio of polyploid formation, such as temperature, genotype, and the origin of the parents (Otto & Whitton, 2000), however the estimations of polyploid formation is 1 every 100 000 individuals (Ramsey & Schemske, 1998). Many proposed pathways are described by which polyploids can originate, like interspecific hybridization followed by chromosome doubling (allopolyploids), fusion of unreduced gametes produced by diploid parents (auto and allopolyploids), and somatic chromosome doubling of meristems (Ramsey & Schemske, 1998; Pelé *et al.*, 2018), although fusion of unreduced gametes from diploid parents is thought to be the main route of polyploid formation (Harlan & de Wet, 1971; Mason & Pires, 2015).

After formation and during early stages, the new polyploid genome has to rapidly adapt to share a single nucleus and to cohabit with duplicated gene copies (and chromosomes), and soon thereafter, start evolving and competing with their related diploid species (Comai, 2005). Polyploids that are able to adapt and overcome extinction start diploidization, that refers to the evolutionary process by which polyploids return to diploid-like organisms with bivalent pairing in meiosis (Wolfe, 2001). The process of diploidization consists of massive gene loss and genome shuffling (Paterson *et al.*, 2004; Chen, 2007). One pair of duplicated gene copies can have different fates depending on the function (Fig. 1 (Birchler & Yang, 2022)). Usually, most genes across angiosperms tend to be lost via a non-random process, with a bias towards single-gene-retention (Thomas *et al.*, 2006; Woodhouse *et al.*, 2010) for those genes involved in maintenance of genome stability (Li *et al.*, 2016a) like DNA metabolism, RNA binding (Freeling, 2009), and meiosis related genes (Lloyd *et al.*, 2014). For example, in *Arabidopsis,* genes preferentially retained encode transcription factors, protein kinases, and ribosomal proteins (Freeling 2009).

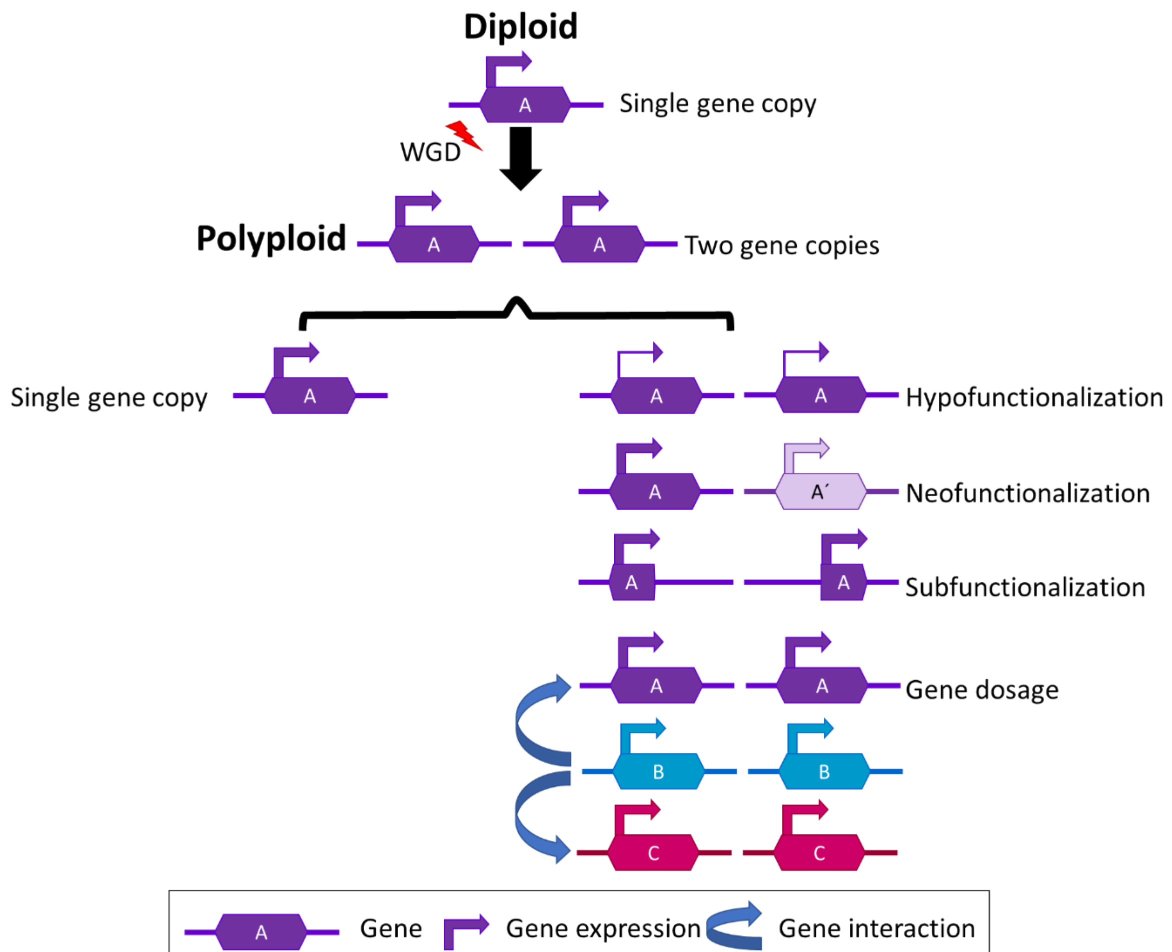


Figure 1. Adapted from (Birchler & Yang, 2022) **The fates of duplicated genes.** Single copy gene present in the diploid parent are shown in purple, gene expression is represented with an arrow, and changes in gene are also drawn with different colors and letters. After a whole genome duplication (WGD) event, the same gene is present twice in a polyploid. One gene copy can be loss, leading to single gene copy (singleton). Both copies can be maintained but gene expression is reduced in both copies (hypofunctionalization). Neofunctionalization occurs when one of the copies has gained a new function different from the original. Subfunctionalization is when both copies retain part of the function of the original gene. Gene dosage can mediate the retention of specific genes that are in a stoichiometric relationship with other gene copies (gene interactions with blue arrows).

Establishment of neopolyploids can be challenging, especially from the reproductive point of view considering that they usually have low fertility (Ramsey & Schemske, 2002) or they exist at lower frequency compared to diploid parents (Levin, 1975). However, becoming a polyploid can also confer important new traits such as biotic and abiotic stress resistances and flowering time variation (Schranz & Osborn, 2000), allowing novel polyploids to colonize different niches

to their diploid progenitors (Chen, 2007) . This is one major reason why polyploidy is considered to be an important evolutionary force and one of the main mechanisms of speciation (Leitch & Leitch, 2008).

1.1.1 Polyploids in agriculture

Polyploid crops tend to be larger and more vigorous than their diploid progenitors, which may be some of the reasons why they have drawn attention in plant breeding and agriculture (Able *et al.*, 2007). Many of the crops consumed daily are natural autotetraploids like potato (*Solanum tuberosum* L., 2n = 4x = 48), kiwifruit (*Actinidia chinensis* Planch., 2n = 4x = 116), and leek (*Allium ampeloprasum* L., 2n = 4x = 32), or are allotetraploids such as bread wheat (*Triticum aestivum* L., 2n = 6x = 42), coffee (*Coffea arabica* L., 2n = 4x = 44), and oats (*Avena sativa* L., 2n = 6x = 42) (Sattler *et al.*, 2016).

Since the discovery of colchicine treatment to induce polyploidy (Nebel, 1937), breeders have tried to take advantage of this technique to either improve or create new crops. In acting dividing cells, colchicine inhibits microtubule formation impeding chromatin separation, forming a polyploid cell (Caperta *et al.*, 2006). There are at least three possible scenarios where plant breeders can use colchicine treatment as part of their breeding efforts : 1) autopolyploid production to take advantage of larger plants and/or organs, 2) restoring fertility in interspecific hybrids (e.g. allopolyploids), or 3) in interploidy crosses to facilitate hybrid production by inducing chromosome doubling in one of the parents (Dewey, 1979).

Despite many efforts, not all crops respond to polyploid induction as expected. Successful polyploid induction depends largely on genome composition, mode of reproduction, starting ploidy level, duration of the life cycle, what part of the plant is being bred for (Dewey, 1979), and colchicine concentration and exposure time (Khan *et al.*, 2023).

If chromosome doubling is achieved, crops generally fall into three phenotypic responses: 1) considerable increase in cell size, with similar amount of cells as in the diploid form, producing as a consequence a larger individual, 2) cell volume increment but a reduction in total cell number, with no obvious change in size compared to diploid parent, and 3) no change in cell size, but changes in fertility may be observed (Dermen, 1940). Other phenotypic changes after

chromosome doubling can include changes in leaf color (Tulay & Unal, 2010), photosynthetic rate (Domínguez-Delgado *et al.*, 2021), flower shape (Samadi *et al.*, 2022), increase tolerance to drought, salt, heat, nutrient deficiency, boron deficiency, and cold (del Pozo & Ramirez-Parra, 2015), which are all possible desired traits to improve in already established cultivars.

Great success in producing new crop types after polyploid induction has been particularly achieved in ornamental plants, with at least 43 new polyploid plant types produced in the spam of 15 years (Manzoor *et al.*, 2019). Polyploid induction has also been applied to medicinal plants, such as spearmint (*Mentha spicata* L.). In here, another compound working as a mitosis-inhibitor (oryzalin) was used, and the resulting hexaploid plants produced almost 50% more essential oils compared to the diploid controls (Bharati *et al.*, 2023).

In cereals, the best-known example of man-made polyploid/hybrid up to date is triticale (*X Triticosecale* Wittmack), a new crop created with the aim of combining superior characteristics of wheat, as a high yielding and great grain quality, and rye, as a source for biotic and abiotic tolerances (Mergoum *et al.*, 2019). Triticale is an allopolyploid crop resulting mostly from a cross between either bread wheat (*Triticum aestivum* L. 2n = 42 = AABBDD) or durum wheat (*T. durum,* 2n = 28 = AABB) and rye (*Secale cereale* L.*,* 2n = 14 = RR) followed by colchicine treatment, producing either an octoploid (2n = AABBDDRR = 56) or an hexaploid triticale (2n = AABBRR = 42) (Mergoum *et al.*, 2009). Throughout the years, triticale has become a well-established crop, with 3.8 million hectares (Mha) being harvested worldwide in the year 2021 (FAOSTATS).

Forage crops have also taken advantage of polyploid induction, such is the case of tetraploid red clover (*Trifolium pretense* L.). Autotetraploid red clover cultivars can be produced chemically (by using colchicine or N_2O) or sexually (unreduced gametes) (Meglic & Smith, 1992). Autotetraploid cultivars outperform diploid cultivars in term of forage yield (Amdahl *et al.*, 2016) and have higher resistance to biotic diseases (Vleugels *et al.*, 2013). However, seed production in autotetraploids is lower compared to diploid cultivars (Vleugels *et al.*, 2015). Future efforts in red clover breeding are generally focused on increasing persistence (longevity of the plant), environmental adaptability, and increase forage and seed yield, with special focus on tetraploid cultivars (Taylor, 2008).

Other examples where hybridization and synthetic polyploidy has played a role in breeding efforts are the triploid sugar beet (*Beta vulgaris* L.) (Kinoshita & Takahashi, 1969), triploid

watermelon (*Citrullus vulgaris* L.), triploid cassava “Sree Harsha” (*Manihot sculenta* “Sree Harsha”) (Sattler *et al.*, 2016), and diverse citrus hybrids (Ollitrault *et al.*, 2020).

However, the number of successful examples of human-made polyploids is rather limited. The main reason why a new polyploid crop is not as successful as anticipated is the lack of genome stability (Lloyd *et al.*, 2014). Loosing chromosomes can greatly affect fertility (seed production) and the overall fitness of a plant. Many of the successful examples mentioned above are crops that are either vegetatively propagated (e.g. ornamental plants), the lack of seed production is a desire trait (e.g. seedless fruits), or seed set is not the main breeding goal (e.g. beets). In light of new and ongoing progress in genomics and technologies associated, much research is needed to try to identify genetic determinants that can contribute to maintain genome integrity in poylploids. This new gain knowledge may be use to widen the utilization of artificial polyploidy and hybridization in agriculture (Mason & Batley, 2015).

1.1.2 Problems with recent polyploids

Established polyploids usually have stable meiosis while new synthetic polyploids are generally consider genomically unstable, mostly due to meiotic errors (Pelé *et al.*, 2017a). For many years, researcher have tried to understand the mechanism behind polyploid stabilization, with results suggesting that the path to meiotic stabilization can vary depending on the polyploid type (allo vs auto) and at the same time, the mechanism between species can also vary (Gonzalo, 2022). Newly synthesized polyploids often show irregularities during meiosis and end up producing gametes which are non-viable or have unbalanced chromosome numbers, producing unbalanced offspring (Ramsey & Schemske, 2002). As a consequence, many of the newly formed polyploids (especially hybrids between different parents) are sterile or are only able to produce a low number of seeds (Mwathi *et al.*, 2020).

Meiosis is an important step in the reproductive life of angiosperms because it separates the sporophytic-diploid phase from the gametophytic-haploid phase (Bhatt *et al.*, 2001). Meiosis has also an impact in evolution by allowing sexual propagation, where meiotic errors create genomic variation where selection can act upon (Leitch & Leitch, 2008). At the basic level, meiosis can be separated into a few main stages: DNA replication, chromosome pairing

(synapsis), crossing over (chiasma formation), and two rounds of chromosome separation (Ramsey & Schemske, 1998). The initial physical link between homologous chromosomes (bivalent structure) also depends on the existence of at least one crossover (CO). This CO corresponds to a reciprocal exchange between homologous non-sister chromatids, producing allelic shuffling that can improve genetic diversity (Mercier *et al.*, 2015), eliminate deleterious mutations, and play a role in DNA repair (Gaeta *et al.*, 2007). During the first round of chromosome segregation the pair of homologous chromosomes separate, and in the second round of chromosome segregation the (identical) sister chromatids are set apart (Mercier *et al.*, 2015).

In allopolyploid plants, it is possible to find two or more ancestrally homologous chromosome sets from different evolutionary lineages: these are referred to as homoeologous (Ramsey & Schemske, 2002). Homoeologous chromosomes are partially homologous, with some differences such us DNA sequence or gene order. In newly synthesized polyploids, non-homologous chromosomes can interact when meiosis is not regulated, leading to the formation of multivalents (more than 2 chromosomes interacting) and univalent (single chromosome), and the consequences of these structures can be observed during anaphase I as an uneven distribution of chromosomes (Figure 2, (Le Comber *et al.*, 2010)). When a chromosome is not pairing during meiosis, it is called univalent chromosome, and during meiosis it can have different fates: they may reach one of the poles in an undivided state; they may stay and form a micronucleus, or they may stay and later get divided into two chromatids (Bremer & Bremer-Reinders, 1954).

It has been also shown that stable meiosis in polyploids can be obtained in a short period of time: in newly synthesized allotetraploid *Arabidopsis suecica* (2n = 36), preferential pairing of homologous chromosomes as bivalents was established after only after three generations (Comai *et al.*, 2003). This supports the idea that the parental species are the source of alleles related to genetic control of chromosome pairing behavior and avoidance of homoeologous pairing during meiosis (Comai *et al.*, 2003). Later on, this idea was further supported in a study in *A. arenosa* where eight meiosis genes were found to be potentially responsible for meiotic adaptation in polyploids (Yant *et al.*, 2013).

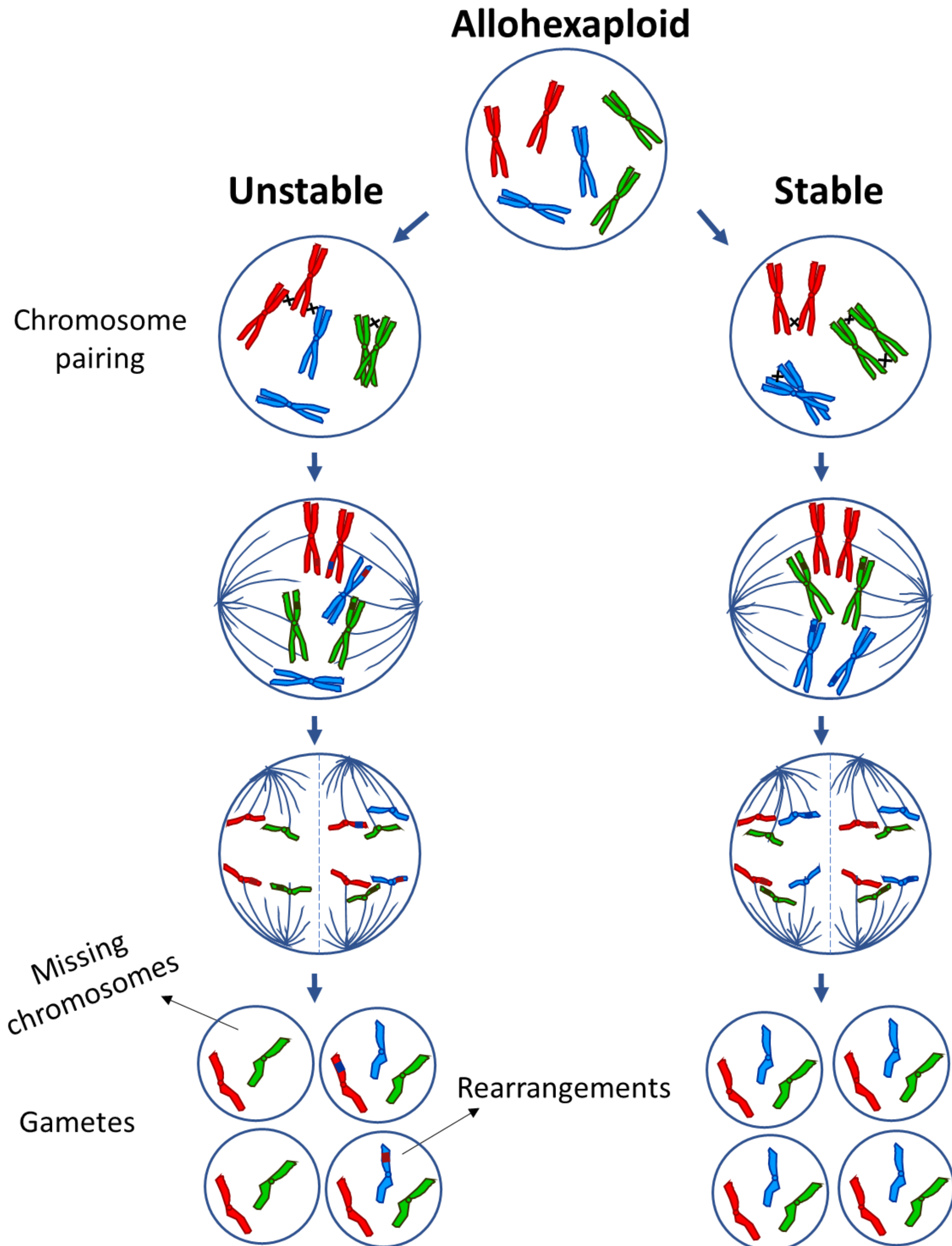


Figure 2. Cartoon representation of meiosis transition in an allohexaploid. For simplicity, only one pair of homolog chromosomes per subgenome are represented in the figure. Each subgenome is shown in a different color: red, green, and blue. On the left of the drawing, simplified unstable meiosis progression is shown. In here, non-homologous pairing and recombination occurs, leading to an uneven chromosome segregation, chromosome loss, and genomic rearrangements present in the gametes. On the left right of the drawing, the normal transition of meiosis is shown, with proper chromosome pairing, recombination, segregation producing gametes with the expected number of chromosomes.

Up to now, a well characterized system for non-homologous pairing of chromosomes during meiosis has been identified in wheat (Riley & Chapman, 1958). In hexaploid wheat (2n = 6x = 42), a locus located on the long arm of chromosome 5B designated as *Pairing homoeologous 1* (*Ph1*) (Wall *et al.*, 1971) is the main determinant in enforcing homologous chromosome pairing during meiosis. *Ph1* locus encodes *ZIP4* (Shen *et al.*, 2012; Martín *et al.*, 2017) and is responsible for promoting synapsis between homologs and the inhibition of crossovers between homoeologous chromosomes (Draeger *et al.*, 2023).

1.1.3 Copy number variation in polyploids

During meiosis, particularly in synthetic polyploids and allopolyploids, chromosomes from different subgenomes can pair and recombine, resulting in non-homologous recombination events (Hurgobin *et al.*, 2018). These events can lead to changes in the genome such as copy number variation (CNV) affecting the stability of the karyotype. Copy number variants are defined as a gain (duplications or extra copy) or loss (missing copy or deletions) of chromosome segments or whole chromosomes that are larger than 1 kb (Figure 3, (Zmieńko et al., 2014)). Several mechanisms have been proposed to explain how CNVs are formed such as non-allelic homologous recombination (NAHR) or non-homologous end joining (NHEJ) (reviewed in (Hastings *et al.*, 2009)). NAHR and NHEJ are DNA repair mechanisms (meiosis context), that in simple terms, can produce CNVs by either using an incorrect template to repair the breaking point (non-allelic region, usually repetitive sequence) or by repairing the DNA damage by joining two DNA ends with low homology (Hastings *et al.*, 2009). CNVs can also happen after a polyploidization event, where some gene copies are subsequently loss, retain, or expanded as part of the diploidization process (Cai *et al.*, 2021).

Different methods have been developed to detect copy number variation such as SNP genotyping array (Yau & Holmes, 2009), real time PCR (Weaver *et al.*, 2010), and next generation sequencing (Zhao *et al.*, 2013). Among these, SNP array is a very affordable technology, that also allows high-throughput genotyping data. CNVs can be identified by looking at the normalize fluorescence intensity data (LogR ratio) obtained from the SNP array. However, SNP arrays also have limitations like marker density and often, deletions are easier

to spot compared to duplications (Rousseau-Gueutin *et al.*, 2017; Mason *et al.*, 2017; Stein *et al.*, 2017; Higgins *et al.*, 2018).

CNVs have been studied in several organisms like wheat (Walkowiak *et al.*, 2020), oilseed rape (Schiessl *et al.*, 2017; Stein *et al.*, 2017), and potato (Iovene *et al.*, 2013) and are particularly prominent in domesticated species (Lye & Purugganan, 2019). Studies comparing related species also showed that differences in gene copy number can provide evolutionary advantages (Suryawanshi *et al.*, 2016). CNVs are also a great source of genetic diversity. For example, gene copy number variation in flowering time genes in a *Brassica napus* diversity panel has been found to be associated with the diversification between growing types (Schiessl *et al.*, 2017). In cotton, changes in copy number of the locus *HPDA-D12* have been shown to affect plant architecture (Ji *et al.*, 2021).

However, CNVs can also be detrimental as it has been observed in humans, with several CNVs being the cause of genomic disorders (Zhang *et al.*, 2009). Changes in copy number can also affect gene expression, disrupting the equilibrium of the genetic networks (Mileyko *et al.*, 2008), affect fertility and viability of gametes (Gaebelein *et al.*, 2019b), so identifying frequency and location may allow a better understanding of genomic rearrangements in plants and how stable or unnstable a genome is.

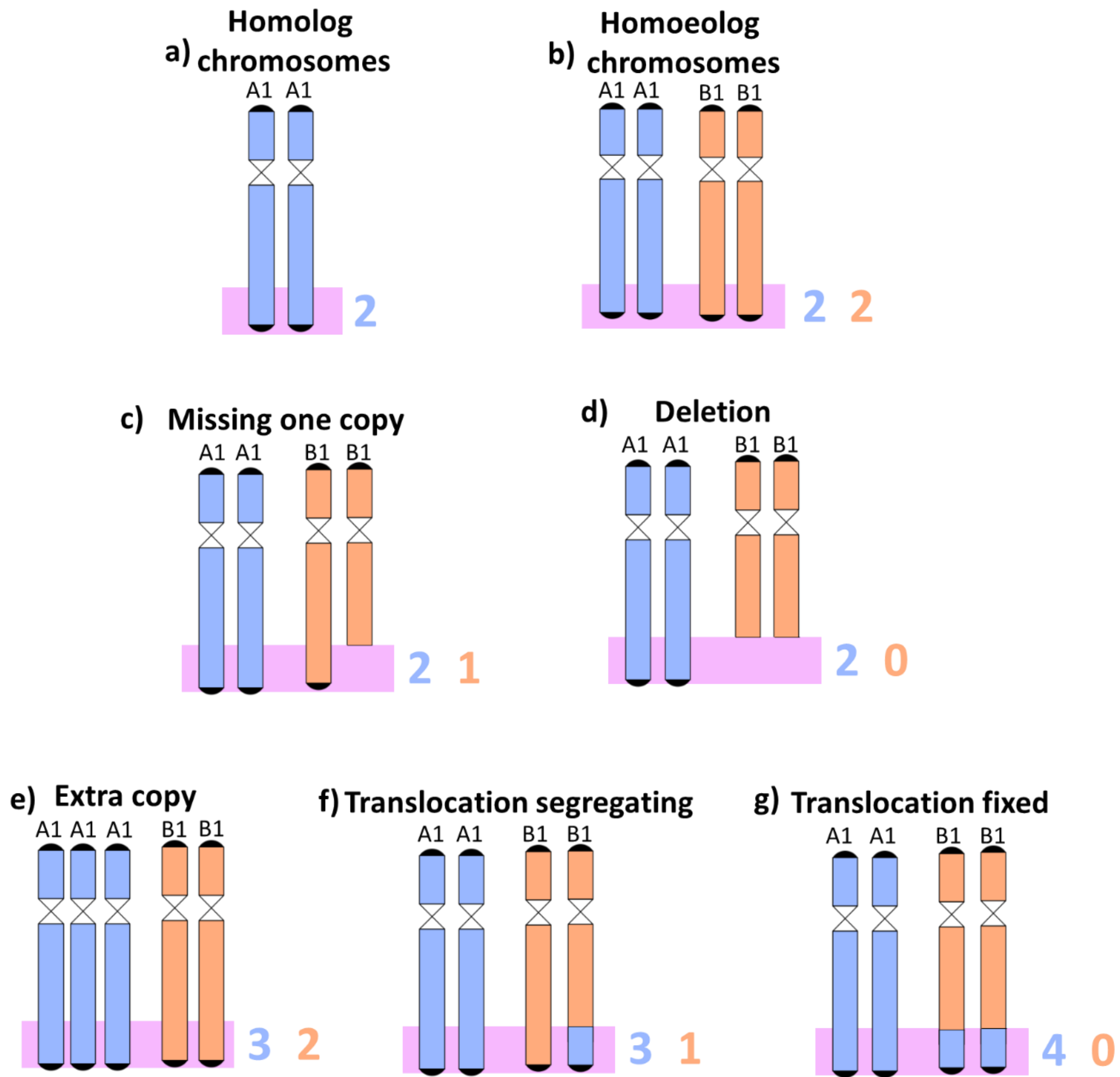


Figure 3. Examples of copy number variation. The region in pink is highlighted to show as an example of copy number variation. a) Homolog chromosomes A1, with the two expected copies (in blue), b) Homoeolog chromosomes A1 and B1, with the expected two copies per chromosome, c) Missing copy event, one homolog region in missing from the B1 chromosome, d) deletion event, both homolog regions are missing in B1, e) extra copy events, in this case there is an extra chromosome for A1, f) translocation segregating (affecting one copy), in here there is a duplicated region from A1 that has been translocated into a missing region in B1, g) translocation fixed, there are two extra copies of A1 translocated into two missing copies of B1.

1.2 *Brassica* genus

The *Brassica* genus belongs to the tribe *Brassiceae* that is part of the Brassicaceae family. This family comprises 338 genera (assigned to 25 tribes) and 3709 species (Al-Shehbaz *et al.*, 2006; Warwick, S I; Al-Shehbaz, 2006). The members of this family are mostly herbs with annual, biennial or perennial growth (Al-shehbaz, 1984). Initially this family was known as "Cruciferae" due to its characteristic flower conformation of four petals arranged in a cross-shape (Al-shehbaz, 1984). Most of the member species are distributed in temperate regions, with the first center of diversification located in the Irano-Turranian region (~150 genera and ~900 species), followed by a second center of diversification in the Mediterranean region (>110 genera and ~630 species) (Al-shehbaz, 1984).

For some of the family members the chromosome number has been established and compiled, with databases covering at least 68.6% of the genera and 42% of the species (Warwick *et al.*, 2006). The lowest Brassicaceae chromosome number is n = 4 in the two non-related genera *Stenopetalum* and *Physaria* (Warwick, S I; Al-Shehbaz, 2006). On the other hand, the highest chromosome number determined is n = 128, belonging to *Cardamine cancatenata* and *C. diphylla* (Warwick *et al.*, 2006).

Brassica is the most prominent genus in the Brassicaceae family and includes 39 species (Warwick *et al.*, 2006). Many of the species in this genus are cultivated for their edible roots, leaves, stems, buds, flowers, mustard, and oilseeds (Rakow, 2004). For 33 of the species the chromosome number has been determined, and ranges from n = 7 up to n = 20 (Warwick, S I; Al-Shehbaz, 2006). During 1930s, the chromosome number and genetic relationships between the six cultivable *Brassica* species was established (Fig. 4 (Nagai, Keizo; Tsunetaro, 1930; U, 1935)). The diploid species *B. rapa* (AA, 2n = 20), *B. nigra* (BB, 2n = 19) and *B. oleracea* (CC, 2n = 18) were determined to be the progenitors of the allopolyploid species *B. juncea* (AABB, 2n = 36), *B. napus* (AACC, 2n = 38), and *B. carinata* (BBCC, 2n = 34) (U, 1935).

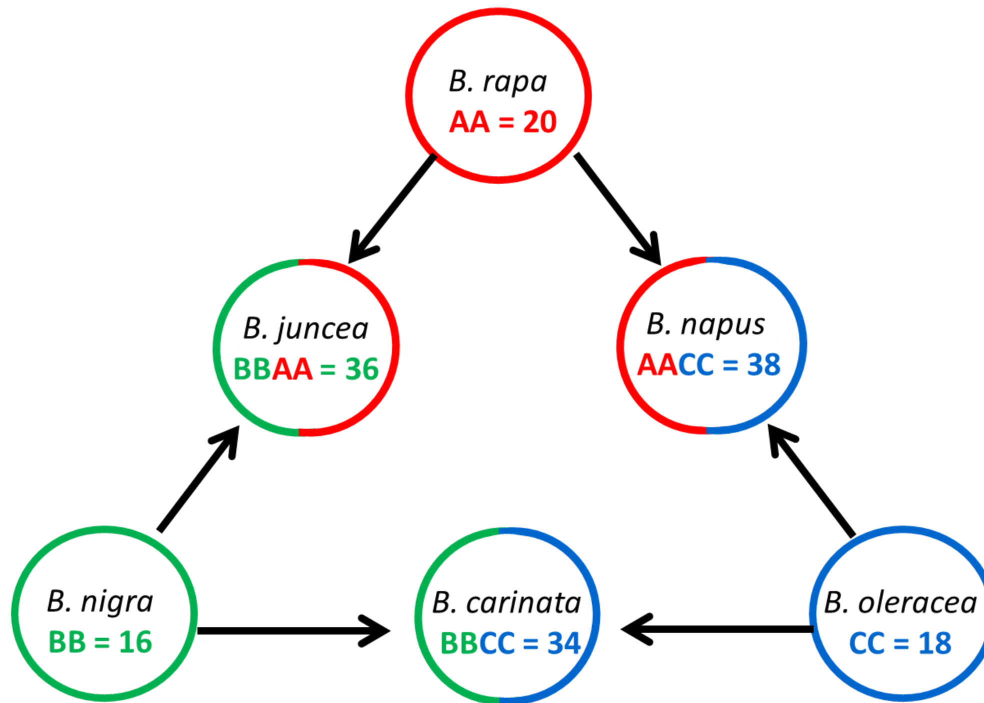


Figure 4. *Brassica* U´s Tringle, the relationship between the six cultivated species (U, 1935). Somatic chromosome number (2n) is represented in each of the species, with *B. oleracea*, *B. nigra*, and *B. rapa* as the diploid progenitors, and *B. carinata*, *B. juncea*, and *B. napus* as the tetraploid species resulting from the pairwise hybridization of two diploid parents, followed by chromosome doubling respectively. Each diploid parent is color, with *B. nigra* in green, *B. rapa* in red, and *B. oleracea* in red. Arrows represent the direction of the cross.

Based on comparative mapping, the *Brassica* genus ancestor was determined to be an hexaploid (Lagercrantz, 1998). Further studies performed in *A. thaliana* (also part of the Brassicaceae family) revealed two ancient whole-genome duplication (WGD) and a triplication (WGT) event is common between *Arabidopsis* and *Brassica* (Bowers *et al.*, 2003; Franzke *et al.*, 2011; Jiao *et al.*, 2012). The oldest WGT event, known as gamma or γ, is thought to have occurred during the Late Jurassic or Early Cretaceous period, around the time of the monocot-eudicot split (Fig. 5 (Jiao *et al.*, 2012)). This WGT event was followed by two other WGD events called β and α (Fig. 5 (Bowers *et al.*, 2003)).Together, these different polyploidization events may have helped in adaptability to the extreme environments present ~65 Mya (Fawcett *et al.*, 2009).

Brassica and *Arabidopsis* lineages split approximately 20 - 43.2 Mya (Koch *et al.*, 2001; Beilstein *et al.*, 2010). After this, *Brassica* underwent an extra WGT event, followed by further species divergence (Nigra and Oleracea/Rapa lineage), and hybridization followed by chromosomes

doubling (Fig.5). The WGT event had a big influence on speciation and expansion, affecting diversity within the *Brassica* genus (Cheng *et al.*, 2014). Three subgenomes have been described as originating from this WGT event in both *B. rapa* and *B. oleracea*. Each subgenome has been named according to the gene density present from high to low: least fractionated (LF), more fractionated 1 (MF1) and more fractionated 2 (MF2). A prevalent characteristic of the LF subgenome is stronger gene expression and fewer non-synonymous mutations compared to the MF1 and MF2 subgenomes (Cheng *et al.*, 2012). After the WGT, the triplicated fragments suffered fractionation and reshufflings within the genome (Cheng *et al.*, 2014) and further chromosome fission/fusion events to reduce the chromosome number (Lagercrantz, 1998).

The *Brassica* lineage has shown high number of rearrangements (90 between *A. thaliana* and *B. nigra*) since the divergence from *Arabidopsis* (Lagercrantz, 1998). Using comparative genomics based on the proposed ancestral karyotype (n = 8), the Brassicaceae genomes can be subdivided into 24 conserved chromosome blocks (labeled A–X) (Parkin *et al.*, 2005; Schranz *et al.*, 2006). Based on this, genomes that have one set of the 24 genomic blocks are considered diploid (e.g. *Arabidopsis*) and those genomes containing more than one set are polyploid (e.g. *Brassica napus*). This new comprehension about the Brassicaceae genomes allows a better comparison between *A. thaliana* and *Brassica* species together with the rest of the members of the Brassicaceae family (Schranz *et al.*, 2006).

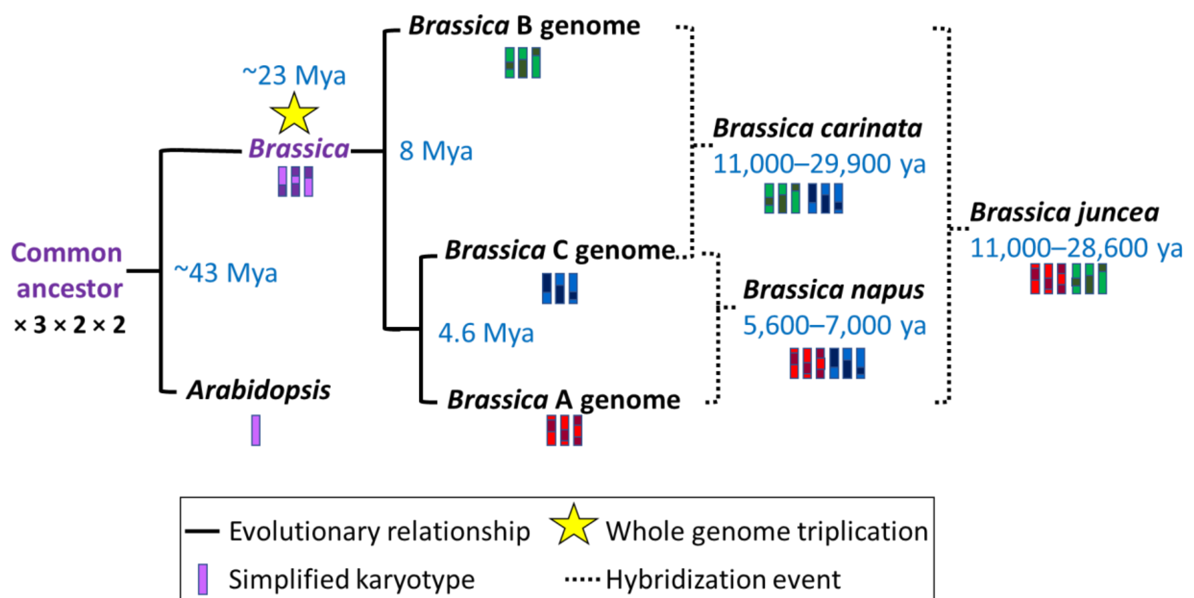


Figure 5. Diagram of the evolution and relationships between *Brassica* U´s triangle species. Estimates of each event are shown in Million years ago (Mya) or years ago (ya) with evolutionary relationships shown as solid lines and hybridization events in dotted lines (see legend). Whole genome triplication event known as γ (× 3) and whole genome duplication events β and α (× 2) that are in common between *Arabidopsis* and *Brassica* are shown (Bowers *et al.*, 2003; Franzke *et al.*, 2011; Jiao *et al.*, 2012). Simplified karyotype with only one chromosome is drawn in the figure. *Brassica* and *Arabidopsis* lineages split ~43 Mya (Beilstein *et al.*, 2010). At ~23 Mya, the *Brassica* lineage underwent a whole genome triplication event (yellow star) followed by genome reshuffling (Beilstein *et al.*, 2010). *Brassica* lineage B genome, also known as Nigra lineage (represented by a green karyotype), diverged from the *Brassica* Oleracea/Rapa lineage (A/C genome) approximately 8 Mya (Lysak *et al.*, 2005). The split between C (represented in blue karyotype) and A genome (red karyotype) occurred 4.6 Mya (Liu *et al.*, 2014). Later on, in the last ~7,000–30,000 years ago, diploid species hybridized in a pairwise fashion (dotted line in the diagram), accompanied by chromosome doubling, to give origin the allotetraploid species *B. carinata* (2n = BBCC), *B. juncea* (2n = AABB), and *B. napus* (2n = AACC (Yim *et al.*, 2022)).

1.2.1 Economic importance of *Brassica* species

In the *Brassica* genus there are six major cultivated species (Fig. 4), with four of them mainly used as oilseed crops: *B. juncea*, *B. rapa, B. carinata,* and *B. napus.* Of these, *B. napus* also known as rapeseed or canola, is the most economically important, occupying the second position as an oil crop in the world. In the year 2021, rapeseed had a production of 71.3 million tons (Mt) (FAOSTATS) with the main producers being Canada, China and India, which together represent almost 60% of the total production world-wide (FAOSTATS). Rapeseed is also used as dairy cow forage (Razzaghi *et al.*, 2022) and is of particular interest in crop rotation systems since it has the ability to enhance soil quality (Zhang *et al.*, 2022), work as a pathogen control (Fang *et al.*, 2016) or affect microbial populations (Li *et al.*, 2021a).

Rapeseed is a self-pollinating annual or biennial crop (Gulden *et al.*, 2008), with three growing types: winter, semi-winter, and spring type. These growing types differ on the requirement of cold exposure to induce flowering, a process known as vernalization (Sheldon *et al.*, 2000). Winter type rapeseed (biennial) requires vernalization to induce flowering and its ideally expose to cold temperatures after reaching a developmental stage of 6-8 true leaf (rosette), has a root collar larger than 5 mm in diameter and a shoot length less than 20 mm (Schröder & Makowski, 1996). Vernalization for winter rapeseed usually last 8 weeks at 2- 12°C and its mainly cultivated in Europe. Semi-winter rapeseed type has mild to no cold requirements to flower and is predominately grown in China (Kumar *et al.*, 2015a). Spring type canola has no vernalization requirement and its mainly grown in Australia and Canada (Kirkegaard *et al.*, 2020). The different types of rapeseed also differ in yield, with winter type canola having a higher yield: 2 – 5 tons per hectare (t/ha), while spring type only reached on average 1.4 t/ha (Kirkegaard *et al.*, 2020).

Rapeseed, as well as other members of the Brassicaceae family, naturally contain 20–40% erucic acid (Mag, 1983) and high glucosinolates in the seed meal (Tripathi & Mishra, 2007). However, rapeseed has been extensively bred for low erucic acid and low glucosinolates (Eskin & Przybylski, 2003) to produce a type of rapeseed better known as canola type or double-low. Unfortunately, as a consequence of this intense breeding, the genetic diversity in canola has been reduced (Fu & Gugel, 2010).

Brassica juncea (L.) Czern & Coss (AABB, 2n = 4x = 36) is a self-pollinated crop mainly grown as vegetable and oilseed, also known as Indian mustard (Kumar *et al.*, 2015a). Based on morphology and purpose, *B. juncea* can be subdivided into four subspecies: *juncea* (seed), *intergrifolia* (leaf vegetable), *napiformis* (turnip-like), and *tsatsai* (stem) (Gladis & Hammer, 1992). *Brassica juncea* is cultivated in North America and South Asian countries, particularly in India, where it has been adapted to the climate (Rai *et al.*, 2022) and it can have a yield potential of 1500 – 3000 Kg/ha (Shekhawat *et al.*, 2012). In China, *B. juncea* is cultivated as a leafy vegetable or turnip, while in Canada its predominantly used as a condiment (Rakow, 2004). Cold conditions are not strictly required to induce flowering in *B. juncea,* however there are some varieties that do require vernalization (Rai *et al.*, 2022).

Brassica rapa (AA, 2n = 2x = 20)*,* initially named *B. campestris* and commonly known as turnip or Chinese cabbage, has its origins in the Mediterranean and Central Asia (Sun, 2015). *Brassica rapa* is an annual and biennial crop, with some types requiring vernalization to flower (Zhao *et al.*, 2007). *Brassica rapa* is an overall outcross species, with the subspecies *trilocularis* (Roxb.) Hanelt, also known as "yellow sarson", as the exception (Gulden *et al.*, 2008).

Brassica rapa has several morphotypes that are cultivated as a fodder (e.g. ssp. *rapifera),* vegetables (e.g. ssp. *chinensis* or *pekinensis*), or as an oilseed crop (e.g. ssp. *oleifera*) (Sun, 2015). Chinese cabbage (ssp. *pekinensis* (Lour.) Hanelt is of particular importance in Chinese, Japanese and Korean cuisine. In Korea, Chinese cabbage is used to produce kimchi (Kim *et al.*, 2014), a fermented product with high monetary revenues (Lee *et al.*, 2017).

Brassica oleracea (CC, 2n = 2x = 18) is mainly used as an edible vegetable, forage and ornamental (Żyła *et al.*, 2021). *Brassica oleracea* has several varieties and morphotypes rich in vitamin C, folate and calcium (Fahey, 2003), resulting from intense breeding and selection from *B. oleracea* wild type (Żyła *et al.*, 2021). Six different categories can be used to separate *B. oleracea* morphotypes: kale (var. *acephala*) as a leafy type; kohlrabi (var. *gongylodes*) with enlarged stem; cabbage (e.g. var. *capitata*) with leaves wrapped around in a head-like shape; inflorescence kales (var. *botrytis,* var. *italica*) such as cauliflower; and Chinese kale (var. *alboglabra*) (Snogerup, 1980). New *B. oleracea* vegetable types have also been produced such as broccolini and anspiration by crossing different varieties within this genus (Fahey, 2003).

The three principal producers of cabbage are China, India and Republic of Korea, while for broccoli and cauliflower the main producers are China, India and United States of America

(FAOSTATS). In the year 2021, the worldwide production of cauliflower and broccoli surpassed 25 million tons, while the production of cabbage surpassed the 71 million tons (FAOSTATS).

Brassica carinata A. Braun (BBCC, 2n = 4x = 34), also called Ethiopian mustard, is an annual self-compatible crop, commonly used as an oilseed crop, vegetable, condiment or medicinal purposes (Alemayehu & Becker, 2002; Rakow, 2004; Zanetti *et al.*, 2013; Kumar *et al.*, 2015b). Similar to other *Brassica* species, *B. carinata* oil is high in erucic acid (35 – 51%) (Alemayehu & Becker, 2001) making it not optimal for human or animal consumption but excellent as a source of biodiesel or even jet-fuel (Redda *et al.*, 2022).

Brassica carinata has its origin in in East Africa and Ethiopia, particularly in Zambia, Kenya, and Tanzania, where its grown and consumed as a leafy vegetable (Ethiopian kale) (Chadha *et al.*, 2007). Limited cultivation has been introduced to other regions such as Australia, Europe, and North America with the main purpose to be tested as a biodiesel crop (Getinet *et al.*, 1996; Cardone *et al.*, 2002; Bouaid *et al.*, 2005; Khangura & Aberra, 2006).

Brassica nigra (L) Koch (BB, 2n = 2x = 16), also known as black mustard, was initially used as a condiment mustard but later on was overtaken by *B. juncea* (Vaughan, 1977; Kumar *et al.*, 2015b). *Brassica nigra* is grown as an annual crop and does not require vernalization to flower (Thomas *et al.*, 2004; De Zoysa & Waisundara, 2020). In recent years, *Brassica nigra* has also been studied as a novel forage crop in ruminants, with promising results (Karydogianni *et al.*, 2022). Furthermore, *B. nigra* is also a great source of resistance to pathogens (Westman *et al.*, 1999) and herbivores (Oduor *et al.*, 2011).

1.2.2 Crossability between *Brassica* species

Plants have developed pre and post fertilization barriers that are aimed to avoid self-pollination or cross-pollination to other species or genera. Self-incompatibility (SI), is a system working against self-fertilization (Sobotka *et al.*, 2000) where self-produced pollen is rejected and foreign pollen is accepted allowing pollen-tube germination and fertilization (Kaneko & Bang, 2014). There are two types of self-incompatibility: gametophytic self-incompatibility (GSI) and sporophytic self-incompatibility (SSI) (Takayama & Isogai, 2005). Gametophytic self-

incompatibility is determined by the haploid gene complement present in the microspores while SSI is determined by the genetics of the diploid plant (rejecting by the stigma) (Nasrallah *et al.*, 1991). *Brassica* species are known to be self-incompatible (particularly diploid species), with sporophytic SI, and is controlled by a single highly polymorphic S-locus (Cabrillac *et al.*, 1999).

Interspecific-incompatibility has been developed in plants to prevent crossings between different species. The genetic basis of this inter species incompatibility is not yet fully understood (Kitashiba & Nasrallah, 2014) and like in SI system, there are pre and post fertilization barriers. In *A. thaliana,* a single gene called *STIGMATIC PRIVACY 1* (*SPRI1*) has been identified to be associated with interspecific-incompatibility (Fujii *et al.*, 2019). In here, the gene *SPRI1* acts as a stigma-specific plasma membrane protein that rejects pollen from other species, working independently from the SI system (Fujii *et al.*, 2019).

Initial attempts to create hybrids between *Brassica* species started in the early 1800s. At this time, some crosses were made between *B. napus* × *B. rapa* and *B. oleracea* × *B. rapa.* Different success rates were reported and the results were published by Kakizaki (1925). Later on, FitzJohn and collaborators published a compilation of crossability between species in the *Brassica*, *Raphanus* and *Sinapis* genera, showing that interspecific hybrids can be made between the *Brassica* crops and many closely-related wild species (Fitzjohn *et al.*, 2007), however, depending on the cross, extra effort may be needed to ensure the formation of a hybrid seed.

Due to the agronomical relevance of interspecific hybridization, scientist have found different methods to overcome pre-fertilization barriers. Pre-fertilization barriers in interspecific hybridization can be overcome through alternative methods. For example, the use of irradiated mentor pollen has been utilize in crosses between *Diplotaxis siettiana* × *B. juncea* (Sarmah & Sarla, 1995). This technique consists in using compatible “mentor” pollen as a stimulus for acceptance of the incompatible pollen, allowing it to germinate and fertilize the ovules (Knox *et al.*, 1987). Application of gibberellic acid before pollination has also been use in interspecific hybridization between *B. rapa* **×** *B. oleracea* (Das *et al.*, 2021). This technique consist in spraying gibberellic acid during anthesis one day before emasculation and pollination, resulting in an increment of crossability between the species (Das *et al.*, 2021). Another way to hybridize different species or genera is by avoiding sexual reproduction and utilizing the fusion of

protoplasts. Protoplasts are somatic cells without the cell wall (Yoo *et al.*, 2007) that are incubated together in a solution that promotes fusion of membranes to obtain a hybrid nucleus (Navrátilová, 2004). Protoplast fusion has been used to produce different types of hybrids in the *Brassica* genus (Ovcharenko *et al.*, 2023), like in the hybridization between *B. oleracea* × *B. rapa* to improve biotic resistance (Ren *et al.*, 2000).

Post-fertilization barriers between interspecific hybridizations can also be overcome by rescuing the developing embryo through for example *in vitro* culture of the ovary, ovule, or embryo (reviewed in (Inomata, 1993)). Ovary culture consists in removing the fertilized ovary few days after pollination, followed by surface sterilization and culture in a suitable medium until seeds develop (Wen *et al.*, 2008). For example, ovary culture has been used to rescue developing embryos in crosses between *B. rapa* and *B. oleracea* (Zhang *et al.*, 2004) or in intergeneric crosses between *Moricandia arvensis* and *B. rapa* or *B. nigra* (Takahata & Takeda, 1990). Another way to rescue a developing hybrid seed is ovule culture, which is often used for rescuing embryos that are aborted early on in the developmental stages (Sarma *et al.*, 2023). The protocol is similar to ovary culture, but in this case, the ovary is cut open to remove the developing ovules that are then placed in a suitable medium to continue growing *in vitro* (Hilgert-Delgado *et al.*, 2015). Ovule culture has been used to produced hybrids between *B. rapa* × *B. oleracea* (Hilgert-Delgado *et al.*, 2015) or in crosses between *B. rapa* and *Raphanus sativus* (Takeshita *et al.*, 1980). Finally, embryos can be rescue from inside the ovules and grown under tissue culture conditions. Embryo culture has been carried out to rescue *B. napus* × *Sinapis alba* hybrid embryos (Ripley & Arnison, 1990).

If the pollen is not rejected by the stigma, the pollen germinates and is able to double fertilize both female gametes to form the diploid embryo and the triploid endosperm (Bleckmann *et al.*, 2014). Even though fertilization can occur, later on it can also lead to embryo abortion (post-fertilization barrier), usually associated with endosperm developmental issues particularly in interspecific crosses (Haig, David; Westoby, 1991). This often happens in one direction (i.e. when one species is used as the maternal parent, but not when it is used as the paternal parent) and it can be overcome when the reciprocal cross direction is tested (Haig, David; Westoby, 1991).

1.2.3 Meiosis in *Brassica*

Meiosis is pivotal in sexual systems, generally consisting of DNA replication, homologous chromosome pairing and recombination, chromosome segregation, and sister chromatid segregation, producing at the end haploid spores (Mercier *et al.*, 2015). Generally, in cultivated diploid *Brassica* species meiosis occur normally, with homolog chromosomes pairing and recombining, followed by correct chromosome segregation (Grandont *et al.*, 2013a). However, in natural *Brassica* tetraploid species, meiosis is more convoluted due to the presence of homoeologous chromosomes, that despite diverging millions of years ago, still maintain sequence similarities and may pair during meiosis (Grandont *et al.*, 2014). For example, in *Brassica napus* we have the homoeologous chromosomes from the A and C subgenome residing in the same nucleus. Because there is sufficient similarity between the A and C genome chromosomes for them to pair during meiosis, cytological diploidization in this species requires that homoeologous pairing is suppressed to ensure proper chromosome segregation (Jenczewski *et al.*, 2003). Nevertheless, it has been shown that in cultivated *B. napus* non-homologous exchanges do still occur, although at a low frequency and it still remains unknown how meiotic stabilization evolved to become stable in natural *Brassica* polyploids (Parkin *et al.*, 1995; Udall *et al.*, 2005; Higgins *et al.*, 2018). When meiosis was analyzed in amphihaploids (AC, n = 19) from crosses between *B. oleracea* and *B. rapa,* a high degree of chromosome pairing and chiasma formation was observed (Attia & Röbbelen, 1986). This study also observed a wide variation in the pairing configurations exhibited, suggesting one or more genetic factors controlling meiotic pairing (Attia and Röbbelen 1986). By analyzing a segregating haploid population made from a cross between a low-pairing (Yudal; few A-C pairs) and a high-pairing (*Darmor-bzh*; many A-C pairs) variety it was demonstrated that the difference in pairing is genetically controlled in *Brassica* (Jenczewski *et al.*, 2003). A major locus responsible for controlling meiotic pairing in haploid *B. napus* (AC) was identified and named *Pairing regulator in B. napus* (*PrBn*) (Jenczewski *et al.*, 2003). Later on, this locus was mapped to the C genome linkage group DY15, on chromosomes C09 (Liu *et al.*, 2006). *PrBn* explains 24% of the variation for the number of univalents in allohaploid *B. napus* (Liu *et al.*, 2006)*,* however the same effect was not observed in tetraploid *B. napus* (Nicolas *et al.*, 2009; Grandont *et al.*, 2014). Other significant quantitative trait loci (QTL) with weak effects have been described in *B. napus*, with one located on the A genome linkage group DY4 explaining ~12% of univalent formation, and

another two with minor effects (Liu *et al.*, 2006). Summing all the individual contributions from the previously four QTLs accounted for 40% of overall variation for the univalent values, suggesting that several other genes also contribute to the prevention of homoeologous pairing in *B. napus* (Liu *et al.*, 2006).

A major QTL controlling homoeologous recombination was recently identified in *Brassica napus,* named *B. napus Pairing homoeologous 1* (*BnaPh1,* (Higgins *et al.*, 2021)). This QTL is located on chromosome A09 and explains 32-58% of the variation in homoeologous recombination observed in a mapping population between stable and unstable *B. napus* (Higgins *et al.*, 2021). Several meiotic genes can be found within the QTL interval that can be potentially studied to elucidate the mechanisms behind *BnaPh1* and meiosis stabilization in *Brassica* polyploids.

1.2.4 Making new polyploid crop types in *Brassica*

Brassica species and relatives are highly versatile, offering great variability in vegetable type, growing type and the possibility to intercross different species to produced new more diverse crop types. For example, diploid *Brassica* species has been utilize to produce new synthetic *Brassica* allotetraploid plats to increate genetic diversity (e.g. (Zou *et al.*, 2018)). At the same time, other new crop types have also been produced by hybridization different *Brassica* species and related genera.

One of the first well known example of attempts to create a new crop type was by crossing *Raphanus sativus* with a diploid *Brassica.* The resulting hybrids were called *XBrassicoraphanus* and *XRaphanobrassica*, and were produced by crossing *R. sativus* (RR, 2n = 18) with *B. oleracea* or *B. rapa* respectively*,* followed by chromosome doubling. These crosses produced what we commonly know as Radicole (CCRR, 2n = 36) (Karpechenko, 1928) or Raparadish (AARR, 2n = 38), respectively (Lange *et al.*, 1989). Both of these hybrids feature a fodder-like crop with the advantage of resistance to the beet cyst nematode. Unfortunately, these hybrids did not produce many seeds (Lange *et al.*, 1989). Later attempts to produced *XBrassicoraphanus* hybrids were more successful with the production of Baemoochae (common name), a new crop

that was developed from the cross between *B. rapa* spp. *pekinensis* and *R. sativus* (Lee *et al.*, 2002). Baemoochae is self-compatible, unlike both of its progenitors, however as we usually observe in new polyploids, the genome is unstable and produced low number of seeds, more likely due to meiotic irregularities (Richharia, 1937; Lee *et al.*, 2002). A more fertile and putatively stable *XBrassicoraphanus* (*R. sativus* × *B. oleracea* var. *albogrovula*) was obtained after 10 generations, showing also great potential as a new fodder crop (Chen & Wu, 2008).

Natural occurring polyploids in *Brassica* are allotetraploids, however autopolyploids can also be chemically induced. However, they do not always show significant advantages compared to the progenitor. For example, production of autopolyploids in *B. oleracea* has been undertaken using two different cultivars (Albertin *et al.*, 2005). This study compared the synthetic tetraploid with the diploid species at the protein level (using 2D-SDS page gels) in the leaf and stem, but observed little change in the profiles obtained. At the same time, the phenotypes from the tetraploids were hardly distinguishable from the diploid parents (Albertin *et al.*, 2005). Nonetheless, successful examples showing advantages of tetraploid vs diploid crop plats do exist, like in a tetraploid turnip rape (*B. oleracea* cultivar "Aijiaohuang") which showed better adaptation to salinity than its diploid progenitor (Meng *et al.*, 2011).

Production of a higher ploidy *Brassica* crop that combines the three subgenomes (hexaploid, AABBCC) has been also attempted (reviewed in (Gaebelein & Mason, 2018)). In agriculture, we have great examples of successful hexaploid crops such as wheat and triticale, although the idea of generating a new allohexaploid *Brassica* crop is promising, many challenges still remain (Zhang *et al.*, 2021).

1.3 *Brassica* allohexaploids

"It is hopelessly difficult to meet the current need for the synthesis and breeding of a new hexaploid species in the genus *Brassica*" (Iwasa, 1964).

The search for a stable trigenomic allohexaploid *Brassica* species (2n = AABBCC) has been part of many studies. Different types of cross-combinations have been tested to produce 2n = AABBCC allohexaploids, with different levels of success (reviewed in (Gaebelein & Mason, 2018)). The most frequently attempted cross is *B. rapa* × *B. carinata*, followed by *B. oleracea* × *B. juncea,* and *B. nigra* × *B. napus*, that are also known as carirapa, junleracea, and naponigra type, respectively (Figure 6). All these previous examples of crosses to produce *Brassica* allohexaploids involve colchicine treatment to double the chromosome number of the initial triploid hybrids produced. A new allohexaploid production method was introduced in 2012 that does not involve the use of any chemical to induce chromosome doubling, and instead relies on the production of unreduced gametes (Mason *et al.*, 2012). This study reports production of a near-allohexaploid (AABBCC, 2n = 50) from the cross combination between (*B. napus* × *B. carinata*) × *B. juncea,* an allohexaploid type also known as NCJ (Figure 4) (Mason *et al.*, 2012).

In the past, many allohexaploids types have been produced, but with the almost exclusive purpose to be use as intermediates to improve elite cultivars rather than to generate a new *Brassica* crop, mainly due to the nature of their unstable meiosis (Chen *et al.*, 2011a). For example, carirapa allohexaploids were used to introgress yellow seed coat into *B. napus* (Meng *et al.*, 1998). Furthermore, *Brassica* allohexaploids have also been used to introgress genetic diversity into *B. juncea* (Wei *et al.*, 2016) and *B. napus* (Zou *et al.*, 2010).

To be able to establish a new *Brassica* allohexaploid crop, three main challenges need to be address: 1) genome stability, 2) proof of sufficient genetic diversity, and 3) agronomic potential (Zhang *et al.*, 2021). Genomic instability (as a result of meiotic errors) accompanied by low fertility, is commonly observed in *Brassica* allohexaploids (Iwasa, 1964). For instance, in 1943, Howard crossed *B. rapa* spp. *chinensis* (AA, 2n = 10) × *B. carinata* (BBCC, 2n = 34) to produce a carirapa allohexaploid (Howard, 1942). In a second generation produced by self-pollination, he was only able to cytologically characterize one plant, which had a chromosome number of 2n = 55. Few cells in metaphase contained 27 bivalents plus one single chromosomes, with cells

also contained ring quadrivalent, univalent, and chromosome bridges were also present. Fertility of four F_2 plants under self-pollination and by cross-pollination between them was analyzed with one cross combination producing seeds at a similar ratio to the parental lines (Howard, 1942). Similarly, in 1950, Mizushima combined *B. carinata* × *B. rapa* spp. *pekinensis* followed by colchicine treatment to produce a carirapa allohexaploid (AABBCC; 2n = 54), showing irregular meiosis and uneven chromosome segregation (Mizushima, 1950), and even though F_1 generation of carirapa hexaploid had an euploid karyotype, in the subsequent generations the progeny showed tendency to produce an increment of aneuploids from one generation to the next, with no tendencies to return to an hexaploid ploidy level (F_5 = 2n = 33-42). Further selection over five successive generations also failed to improve fertility, leading to the conclusion that the production of a *Brassica* hexaploid was “hopelessly difficult” (Iwasa, 1964). After several trials, in 2016 was reported the first meiotically stable *Brassica* allohexaploid (carirapa, (Gupta *et al.*, 2016)). The hexaploids were generated by crossing different accessions of *B. carinata* (as the mother) with *B. rapa* (as the father) followed by colchicine treatment of the F_1 hybrids. From all the different combinations tested, two produced plants with a high frequency of bivalent formation and proper chromosome assortment during meiosis. Even more interestingly, both cross-combinations had the same *B. rapa* parent: R01. These plants had the expected 54 chromosomes and no major translocations were observed by GISH, although graphical genotyping suggested that there were some fragment exchanges between the three genomes (Gupta *et al.*, 2016). This provided evidence that the *B. rapa* genotype R01 has a genetic background that contributes to stabilize the new allohexaploids, but unfortunately until now no specific major gene/s have been discovered mediating this effect. However, this study sets the precedent that meiotic stability in allohexaploids is achievable and that there is potential to establishing *Brassica* allohexaploid as a new crop type.

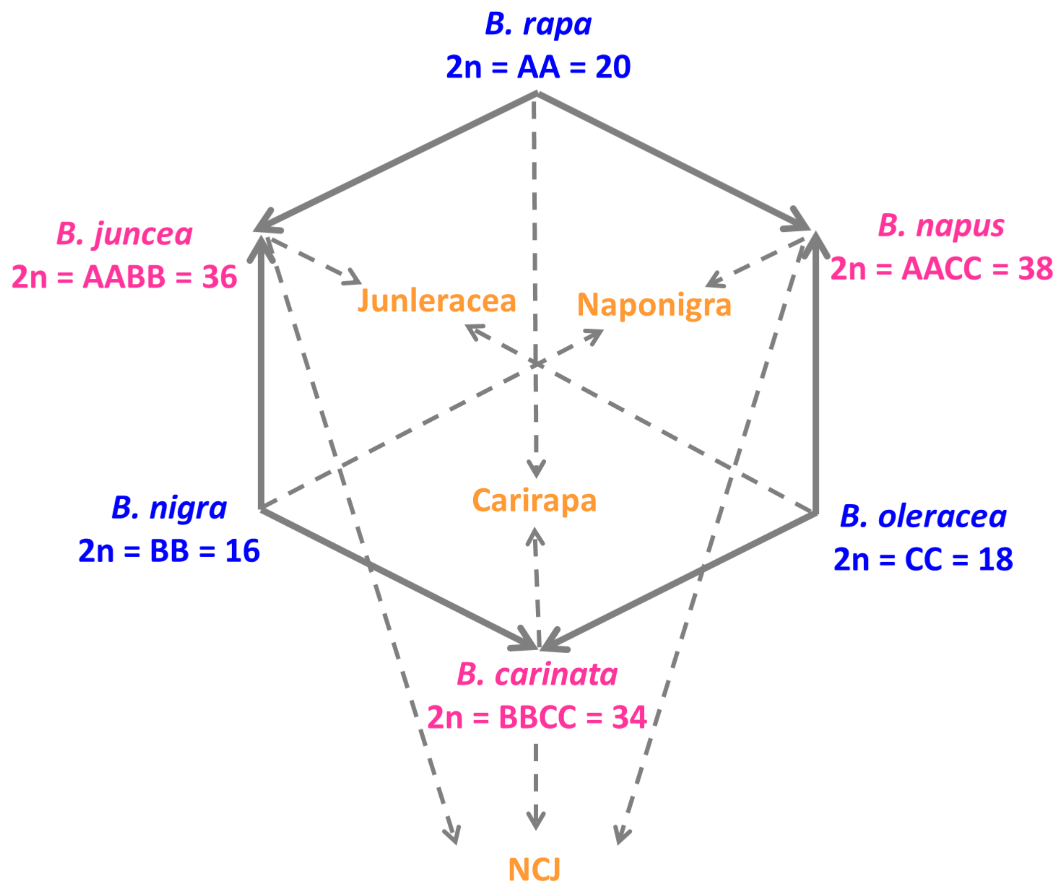


Figure 6. Crossing scheme for the different *Brassica* types. Diploid species (colored in blue) hybridized to originate the allotetraploid species (colored in pink). The direction of the cross between diploid species is drawn in solid gray arrows. Crossings between diploids and tetraploid species used to produce the allohexaploid types junleracea (*B. juncea* × *B. oleracea*), naponigra (*B. napus* × *B. nigra*), NCJ (*B. napus* × *B. carinata* × *B. juncea*), and carirapa are colored in orange (AABBCC = 2n = 54) and the crosses between the species that give origin to the allohexaploids are shown with dashed gray arrows.

Further studies have been conducted to try to understand the genetic background of meiosis stability and fertility in *Brassica* allohexaploids. For instance, a high density genetic map was produced from a double haploid population derived from the F_1 hybrid carirapa × naponigra (Yang *et al.*, 2018). In this population it was found that pollen viability was not associated with number of seeds produced (fertility). On the other hand, loss of a whole or part of chromosomes negatively influenced pollen viability. Several QTLs were also identified related to six different phenotypes: seed number, seed yield, plant height, 1000-seed weight, pollen viability, and pod length (Yang *et al.*, 2018). Unfortunately, no meiotic analysis was performed

in this population. Gaebelein et al., (Gaebelein *et al.*, 2019b) analyzed three segregating NCJ allohexaploid populations and found several genomic changes, particularly between the A and C subgenome that affected fertility. At the same time, this analysis also provided a series of potential meiotic candidate genes associated with fertility that can be asses in future experiments (Gaebelein *et al.*, 2019b) to try to further achieve genomic instability in *Brassica* allohexaploids.

The second main challenge to produce a *Brassica* allohexaploid crop is sufficient genetic diversity. In 2010, several different carirapa allohexaploids crosses were attempted using a variety of *B. rapa* and *B. carinata* accessions (Tian *et al.*, 2010). More than 40% of the carirapa hexaploids produced derived only from three different combinations. This example highlights the importance of genetic background in the production of hybrids and at the same time, the difficulties behind creating this material and how limited is the genetic diversity available of successfully produced allohexaploids. Without sufficient genetic diversity, it is also difficult to select for beneficial alleles and further efforts need to focus on either creating new material or combining the existing one.

Finally, the last main challenge to produce a *Brassica* allohexaploid is the proof of agronomic superiority compared to existing crops. New polyploids can exhibit phenotypes different from those present in the progenitors (Song *et al.*, 1993; Abel *et al.*, 2005), proving potentially new vegetable types or a new crop type nonexistent in the genus before. At the same time, many of the main agronomical traits are quantitative traits such as yield and yield-related traits (Zhao *et al.*, 2016) and by combining a whole genome instead of chromosomic regions, the expression of complex traits can be achievedFuture production a *Brassica* allohexaploids should be tailored to the specific agronomical need, as to use the appropriate starting parental material in the crosses as well as the proper assessment under field conditions.

Other allohexaploids from the *Brassica* genus have been produced which include species outside of the U´s triangle. An example of this is the allohexaploid produced by the cross between the three cultivated allotetraploid *Brassica* species (*B. carinata, B. juncea,* and *B. napus*) with *B. maurorum* (MM, 2n = 16) (Yao *et al.*, 2012). This species of *Brassica* has some very attractive traits such as resistance to white rust and Alternaria blight (Chrungu *et al.*, 1999). Initially the triploid hybrids produced were completely male and female sterile, but after chromosome-doubling they regained fertility to a certain extent. The cross combination

between *B. napus* and *B. maurorum* (AACC.MM, 2n = 54) was completely male sterile. The other two combinations *B. maurorum* × *B. juncea* (MM.AABB, 2n = 52) and *B. carinata* × *B. maurorum* (BBCC, 2n = 50) showed higher pollen stainability with 71% and 21%, respectively. These results coincided with the meiotic analysis, where the combination MM.AABB displayed a much more regular meiosis pairing, with the majority of pollen mother cells (PMCs) analyzed showing a meiotic configuration of 26 bivalents with rare occurrence of multivalents (Yao *et al.*, 2012). Even though these plants represent a great opportunity to improve other *Brassica* crops, much more work and selection has to be done to secure stability in further generations.

Overall, there is a lot of potential in producing a new *Brassica* allohexaploid crop type. However, much research is needed into understanding the consequences of hybridization and polyploidization through an assessment of genomic stability and fertility in the material. At the same time, selection for potential stable material is also imperative, due to the low frequency of euploids present (Tian *et al.*, 2010; Mason *et al.*, 2012; Mwathi *et al.*, 2020). Once putatively stable material has been obtained, proof of the agronomic potential of *Brassica* allohexaploid should be done customized to current biotic and abiotic needs in nowadays agriculture.

2. Using wild relatives and related species to build climate resilience in *Brassica* crops.

2.1 Abstract

Climate change will have major impacts on crop production: not just increasing drought and heat stress, but also increasing insect and disease loads and the chance of extreme weather events and further adverse conditions. Often, wild relatives show increased tolerances to biotic and abiotic stresses, due to reduced stringency of selection for yield and yield-related traits under optimum conditions. One possible strategy to improve resilience in our modern-day crop cultivars is to utilize wild relative germplasm in breeding, and attempt to introgress genetic factors contributing to greater environmental tolerances from these wild relatives into elite crop types. However, this approach can be difficult, as it relies on factors such as ease of hybridization and genetic distance between the source and target, crossover frequencies and distributions in the hybrid, and ability to select for desirable introgressions while minimizing linkage drag. In this review, we outline the possible effects that climate change may have on crop production, introduce the *Brassica* crop species and their wild relatives, and provide an index of useful traits that are known to be present in each of these species that may be exploitable through interspecific hybridization-based approaches. Subsequently, we outline how introgression breeding works, what factors affect the success of this approach, and how this approach can be optimized so as to increase the chance of recovering the desired introgression lines. Our review provides a working guide to the use of wild relatives and related crop germplasm to improve biotic and abiotic resistances in *Brassica* crop species.

2.2 Climate change will result in a higher frequency of extreme weather events and increased pest and disease loads

Increasing concentrations of greenhouse gases resulting from industrial activity drive global warming via the greenhouse effect (IPCC, 2014). Average temperatures are therefore rising globally, and are to date about 1 °C on average higher compared to pre-industrial levels (IPCC, 2018), and about 1.5 °C higher over land (Shukla *et al.*, 2019). Global temperatures will continue to rise a further 0.4 °C - 2.6 °C until 2050 depending on various climate protection policies (IPCC, 2014). As a primary effect, rising temperatures increase the likelihood of heat waves (Shukla *et al.*, 2019). Heat stress has negative impacts on plant growth due to its devastating influence on cell membranes and protein stability, and limits plant growth at all developmental stages, but particularly during flowering (Bita & Gerats, 2013; Bailey-Serres *et al.*, 2019). On top of direct effects, rising temperature can have two further adverse secondary effects on local climates: at warmer temperatures, the water holding capacity of the air increases about 7 % per °C, which can lead to stronger single rain events and increase the likelihood of flooding (Trenberth, 2011; Kodra *et al.*, 2020). At the same time, rising transpiration can dry down soils more quickly and increase the likelihood of droughts (Trenberth, 2011; Lu *et al.*, 2019). Which outcome is more probable depends on season and geography. Central Europe, for example, can expect more rain in the winter season, but more drought in spring and early summer (Lu *et al.*, 2019). Flooding leads to a loss of oxygen in the soil, which in turn leads to denitrification and ionic toxicity. Moreover, depending on how much of the plant is covered by water, flooding can also inhibit gas exchange and photosynthesis and therefore heavily impact plant metabolism (Sasidharan *et al.*, 2018). Drought, on the other hand, leads to a loss of cell turgor, to which most crops react with closure of stomata (Iwaya-Inoue *et al.*, 2018). This inhibits gas exchange and therefore leads to a loss in photosynthetic capacity (Chaves *et al.*, 2009), with the production of reactive oxygen species as a negative side effect (Choudhury *et al.*, 2017). Some farmers try to balance drought by increased irrigation when water resources are available, although this carries the risk of lowering ground water level and causing secondary salinification. The area of saline soils is also increasing, mostly due to unsuitable irrigation practices (Shukla *et al.*, 2019), but also due to rising sea levels as a result of the ice shield melting and expansion of the oceans due to the warmer temperatures (Nerem *et al.*, 2018; Cheng *et*

al., 2020). Salinity negatively affects plant growth and survival, causing osmotic stress and ion toxicity (Chaves *et al.*, 2009).

Finally, there are also tertiary effects of global warming. As climate zones start to shift (Shukla *et al.*, 2019), insects and pests expand their climatic niche into higher latitudes and start spreading towards areas that were previously too cold for them (Suzuki *et al.*, 2014). Moreover, increased abiotic stresses may weaken plant defense mechanisms against biotic stress (Suzuki *et al.*, 2014).

The only putatively positive effect of rising industrial carbon dioxide levels is the fertilization effect via increased efficiency of the dark reaction of photosynthesis (Shukla *et al.*, 2019). However, utilization of this effect depends on plant nitrogen and phosphorus availability (Sinclair *et al.*, 2019) and is therefore mostly only expected in high-input farming. Moreover, the effect is expected to rapidly saturate due to the limited availability of RubisCO (Sinclair *et al.*, 2019), such that additional rises in carbon dioxide are not going to increase growth further.

To summarize, the conditions for plant production are worsening quickly, and the available farm land is decreasing at the same time. Meanwhile, the global population is still rising, and we need to produce more food from less land and worse conditions than ever before. Therefore, crops need to be bred to produce more yield– we need to increase breeding gains. The major prerequisite for breeding gains is, however, genetic variation. In some crops, recent bottlenecks in breeding history have dramatically decreased genetic diversity within the gene pool, with *Brassica napus* (rapeseed) being a particular concern (Snowdon & Luy, 2012). In this review, we introduce how *Brassica* wild relatives and the close relationships between crop species can be exploited to widen genetic diversity and improve resistances to biotic and abiotic stresses in this important group of crops, and outline potential methodology and considerations to using this approach in applied breeding programs.

2.3 The use of wild relatives and related species for crop improvement in *Brassica*

The Brassicaceae, also referred to as the mustard family or the Cruciferae, are a family of flowering plants comprising 338 genera and 3709 species (Al-Shehbaz *et al.*, 2006; Warwick *et al.*, 2006). The Brassicaceae contains several species of research interest, including the model plant *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), as well as crops such as *Raphanus sativus* (radish), *Eruca sativa* (rocket), *Sinapis alba* (mustard seed), and *Brassica napus* (rapeseed). Some species such as *Aurinia saxatilis* (basket-of-gold), *Iberis sempervirens* (candytuft), *Matthiola incana* (stocks), *Erysimum cheiri* (wallflowers) and *Lunaria annua* (honesty) from this family are cultivated as ornamentals. The Brassiceae tribe is one of the 49 tribes in the Brassicaceae family, and is a group containing a number of phylogenetic lineages originating from a single clade. The Brassiceae contains species of various ploidy levels, with chromosome numbers for 80% of the species in this tribe ranging from n = 6 to n = 75 (Warwick & Anderson, 1993). The genus *Brassica*, in the Brassiceae, is made up of 37 species and is the most agronomically significant genus in the Brassicaceae tribe, and has undergone extensive domestication (Gomez-Campo, 1980). This genera includes mainly herbaceous plants believed to have originated from the Mediterranean region, and modern adapted cultivars have a global distribution as cultivated vegetables and oilseed crop plants (Fahey, 2003). *Brassica* crops are commonly consumed as leafy (Pak choy, kale), stem (wasabi) and root (turnips, radish, rutabaga) type vegetables, spice crops (black or brown mustard), cooking oil (rapeseed) and feed for livestock. Next in agronomic significance from the mustard family are Raphanus and Sinapis, which are also useful as edible roots and condiments respectively (Rakow, 2004). Owing to their closeness as members of the same Brassicaceae family, *Brassica* species benefit from the numerous molecular genetics and genomic tools available to Arabidopsis (Snowdon, 2007; Mason & Snowdon, 2016). The close relationship between species of the *Brassica* genus combined with the ample wild relatives and minor crop species in the wider Brassicaceae tribe make it an interesting model for examining interspecific hybridization for crop improvement (Katche *et al.*, 2019).

The Triangle of U, developed by Korean cytogeneticist Nagaharu U (U, 1935), shows the evolutionary and chromosomal relationships between the A, B and C genomes of the diploid species *B. rapa* (AA, 2n = 20; turnip rape, turnip, Chinese cabbage, Pak choi), *B. nigra* (BB, 2n =

16; black mustard) and *B. oleracea* (CC, 2n = 18; cabbage, cauliflower, broccoli, kale, kohlrabi, Brussels sprouts), and their allotetraploids *B. carinata* (AABB, 2n = 34; Abyssinian or Ethiopian mustard), *B. napus* (AACC, 2n = 38; oilseed rape, spring rape, swede) and *B. juncea* (BBCC, 2n = 36; Indian or brown mustard) which were generated through spontaneous interspecific hybridization events between the diploid species. *Brassica napus* is a relatively young crop (< 10 000 years old) which originated from the spontaneous hybridization between turnip rape (*Brassica rapa*; AA, 2*n* = 20) and cabbage/kale (*Brassica oleracea*; CC, 2*n* = 18) (Chalhoub *et al.*, 2014). *Brassica rapa* (n = 10, A genome) originates from the highlands near the Mediterranean sea from where it migrated northward into Scandinavia and westward into eastern Europe and Germany (Nishi, 1980). According to various authors, *Brassica oleracea* (n = 9, C genome), (characterized with distinct phenotypes (Snogerup, 1980)), is believed to be a seaside plant of northern European or Mediterranean origin. Wild *B. oleracea* varieties still exist on maritime cliffs and continue to grow along the coasts of northern Spain, western France, southern and southwestern Britain (Vaughan, 1977; Fahey, 2003). *Brassica carinata* has been cultivated in Ethiopia and neighbouring territories from ancient times, while many researchers agree that *B. juncea* is a plant of Asiatic origin, with Asia as a centre of major diversity (Chen *et al.*, 2013).

Rapeseed, oilseed rape or canola (Canadian Oil Low Acid) is the third most important oilseed crop in the world. Oilseed rape generally refers to any member of the *Brassica* genus which is grown for edible oil (normally *B. napus*, *B. rapa* and *B. juncea*), while rapeseed technically refers just to *B. napus*. Rapeseed attained economic importance as a source of edible vegetable oil after intensive breeding programs that led to the production of lines with low erucic acid (<2% in the oil), low glucosinolate content (<30 mg/g in the meal) and increased yields. All these breeding efforts and intensive selection for agricultural purposes have led to the generation of elite varieties with low genetic diversity compared to the wider gene pools (Snowdon & Luy, 2012). *Brassica napus,* via human migration, went from Europe (where it first originated) to other parts of the world because of its usefulness as a high yielding *Brassica* crop with high seed quality (Zou *et al.*, 2010). Winter rapeseed first spread to Russia, then to Japan and later on to China, while spring rapeseed reached China via Canada (Wu *et al.*, 2019). Presently, almost 60 % of the total global rapeseed production is from Canada, China and India (www.fao.org/faostat/November 2018), with the EU and Australia as other major rapeseed producers. In addition to serving as a good source of edible vegetable oil, rapeseed is also a

valuable animal feed ingredient for ruminants and monogastric farm animals, used in producing industrial compounds like lubricants and surfactants and also as a raw material for biofuels in diesel cars and tractors, mostly in Germany and Europe (Allender and King 2010; Zou et al. 2010; Friedt et al. 2018). Qualities that make canola the preferred choice of oil by nutritionists and consumers around the world include its high content of poly-unsaturated linolenic acid (richness in omega-3, ca. 10%) and high content of oleic acid, ca. 60% (Iniguez-Luy & Federico, 2011; Friedt *et al.*, 2018). However, the balance of uses in the brassicas need to be maintained as the value of the vegetable brassicas is outstripping *B. napus* globally especially in light of losses due to insects since the removal of chemical controls by the European Union.

2.4 Useful traits identified in *Brassica* crops and wild allies

Each of the six major cultivated *Brassica* species contain unique, potentially useful agronomic traits that can be utilized to improve elite cultivars or to increase the gene pool within a species. While each species is often strongly associated with a particular phenotype, e.g. such that *B. napus* is widely known as a high yielding oilseed crop (76 MT produced in 2007, FAOSTATS) and *B. oleracea* as a highly variable vegetable type (Cheng *et al.*, 2016), many traits present in individual species can be transferred between these closely related species for crop improvement. In the year 2009, a compendium of known traits in *Brassica* and wild relatives was published (Warwick, 1993). Since then, many other genotypes carrying relevant traits for agronomic improvement have been found in different *Brassica* accessions.

2.4.1 Insect resistance traits

Insects also are a big problem in *Brassica* crops and major yield losses and aesthetic damage can occur under their attack. The major pests attacking *Brassica* belong to the order of Lepidoptera, Hymenoptera, Diptera, Homoptera and Coleoptera (reviewed in (Ahuja et al.

2011)), many of them with the ability to move and migrate to infest their hosts. A very common oilseed rape pest is the pollen beetle (*Brassicogethes aeneus,* previously known as *Meligethes aenus*), that can cause more than 80% yield losses (Hansen, 2004). Unfortunately, to date, no natural resistance has been found and the only way to protect the plants is through insecticide application or other integrated pest management strategies. Due to this, new resistant insects have emerged (Spitzer *et al.*, 2020) and novel strategies are required to control the pest (reviewed in (Hervé & Cortesero, 2016)). For other pests, such as Diamondback moth (*Plutella xylostella*), that can cause severe economic damage (Zalucki *et al.*, 2012; Li *et al.*, 2016b), resistance has been observed in a single line of *B. oleracea* spp. *capitata* (Kim *et al.*, 2013).

In 2015, 432 different accessions of *B. oleracea* and allies were tested against cabbage whitefly (*Aleyrodes proletella* L.), out of which 48 showed a high degree of resistance (Pelgrom *et al.*, 2015). In this study, the wild relatives *B. incana, B. montana* and *B. villosa* were shown to be very unappealing to the pest under early growth and development conditions. One possible explanation for the observed resistance in *B. incana* is the presence of trichomes (absent in the susceptible genotype).

The pest known as cabbage seedpod weevil (CSW; *Ceutorhynchus obstrictus*) severely affects oilseed rape, especially during the early flowering period (reviewed in (Dosdall, 2009)). Resistance for this pest has been found in lines produced by the cross of *Sinapis alba* (resistant parent) and *B. napus* (susceptible parent) (Tansey *et al.*, 2010; Lee *et al.*, 2014). Resistance to another weevil pest, *Ceutorhynchus napi*, also known as rape stem weevil, was found in resynthesized *B. napus* lines (Schaefer-Koesterke *et al.*, 2017). The resistance observed might be due to antixenosis (non-preference) given the extended size of the stem and also the lack of specific glucosinolate compounds (Schaefer-Koesterke *et al.*, 2017).

Fully developed cabbage root fly (*Delia radicum* L.) infests its host by laying eggs on the ground, close to the plant, where the larva can live by feeding from the roots, therefore affecting plant development and eventually damaging yield loss (Hopkins *et al.*, 1999). In a panel composed of diverse *Brassica* species, the antibiosis resistance (adverse effects on the pest) of these plants against cabbage root fly was studied (Shuhang *et al.*, 2016). Here they found high levels of antibiosis in *B. spinenscens* and *B. fruticulosa* under greenhouse conditions, given by the observed fewer eclosed flies per egg and reduced fly dry weight (Shuhang *et al.*, 2016). Other potential resistance candidates more readily crossable with *Brassica* crops are accessions found

in *B. montana, B. macrocarpa, B. villosa, B. hilarionis* (Shuhang *et al.*, 2016) and *B. rapa* (Santolamazza-Carbone *et al.*, 2017).

In a 2-year case study, *Eruca sativa* cv. T 27 followed by *B. carinata* cv. DLSC 2 were the least infested by aphids (*Lipaphis erysimi*) under normal conditions when compared to *B. juncea, B. rapa* and a hybrid *B. napus* (Kumar & Sangha, 2017). Between the species studied, there were different chemical profiles present in the inflorescence that can explain over 94% of the amount of aphids present (Kumar & Sangha, 2017). Screening for resistance to the moth *Mamestra brassicae* was carried out in 21 cabbage (*B. oleracea* var. *capitata*) varieties (Cartea *et al.*, 2010). The two more resistant varieties had the compact head characteristic, a morphological trait that can also be involved in insect resistance (Carmona *et al.*, 2011). Some of the insect pests affecting Brassica plants can work as carriers of other diseases like viruses. There are several viral infections described as affecting *Brassica* crops, especially cabbage types, including for example cauliflower mosaic virus (CaMV), turnip yellow mosaic virus (TyMV) and turnip mosaic virus (TuMV) (Raybould *et al.*, 1999). A combination of TuMV and CaMV infection can affect up to 25% of the yield in *B. oleacea* var. *capitata*, mostly due to TuMV as no significant effect was observed when CaMV was inoculated alone (Spence *et al.*, 2007) and in current times most of the research has focused on identifying resistance for TuMV. Turnip mosaic virus (TuMV) infections in crucifer plants was initially described in 1921 (Schultz E. S., 1921), where the characteristic spotted pattern of a "mosaic like virus" was observed in *Brassica rapa.* This disease is mainly transmitted by aphids and non-exclusively infects *Brassica* genotypes (Walsh & Jenner, 2002; Shattuck, 2010), causing a reduction in fitness, reproduction and quality of the plant (Maskell *et al.*, 1999). The utilization of insecticides against aphids to control the spread of TuMV is not very efficient, consequently the identification and utilization of natural resistant *Brassica* varieties becomes the prefer option to control the disease in an environmental friendly way (Walsh *et al.*, 1999).

In one study, *B. juncea*, *B. oleracea, B. rapa, C. sativa* and *R. sativus* lines were tested against TuMV virus pathotype 8 (Nyalugwe *et al.*, 2015): different *B. oleracea* and *R. sativus* lines showed consistently extreme resistance to the virus. The rest of the lines showed different responses to the infection although there was potential for resistance in each of the species tested (Nyalugwe *et al.*, 2015). Also in this study, a dominant gene conferring systemic resistance in *B. juncea* was identified (*TuMV RESISTANCE IN BRASSICA JUNCEA 01*) (Nyalugwe

et al., 2015, 2016). Extreme resistance to the TuMV pathotype 8 has been also observed in 18 *B. napus* and 14 *B. carinata* lines from different origins (Nyalugwe *et al.*, 2014). A resistance to TuMV virus found in *Raphanus sativus* was identified and successfully transmitted via somatic fusion with *B. oleracea* var. *capitata*, *B. oleracea* var. *botrytis, B. oleracea* var. *capitata*, to 61, 83.6 and 33.2% of the hybrids produced, respectively (Scholze *et al.*, 2010).

2.4.2 Disease resistance traits

Disease resistance has been broadly studied due to the major impact on crop production and yield. Resistance to a particular disease can be governed by a single gene (e.g. an "R-gene") or by many genes with minor effects (quantitative resistance). Although many *Brassica* cultivars have been identified to carry particular disease resistances, pathogen evolution rapidly overcomes individual resistance sources or types under the high selection pressure of cropping production systems, such that the need for new resistance alleles is an ongoing process. Clubroot (CR) disease caused by many identified pathotypes of pathogen *Plasmodiophora brassicae* is prevalent around the world and greatly affects production in *Brassica* cultivars (Dixon, 2009). Major resistance to CR has been found, for example, in *Brassica rapa* (Karling, 1968; Piao *et al.*, 2009; Zhang *et al.*, 2015a). A large-scale screening for CR resistance against pathotype 3 carried out in a collection of 955 *Brassica* accessions (mostly *B. rapa*), revealed highly resistant accessions of *B. rapa* (17)*, B. nigra* (4), and *B. oleracea* (2) (Peng *et al.*, 2014). Another screening test of 22 CR isolates against 386 *Brassica* accessions (between 63-65 accessions of each species *B. rapa, B. nigra, B. oleracea, B. napus, B. juncea,* and *B. carinata*) revealed that most resistance sources were present in *B. nigra,* with some in *B. oleracea*, *B. rapa* and *B. napus* (but none identified in *B. juncea* or *B. carinata*) (Fredua-Agyeman *et al.*, 2019). Resistance to CR and downy mildew (*Peronospora parasitica* subsp. *brassicae*) was also tested in 52 accessions of *B. oleracea* and revealed frequent resistance to powdery mildew but only few lines possessed CR resistance (Carlsson *et al.*, 2004). Further studies have also found field-based resistance to downy mildew in serveral *B. oleracea* lines (Monot & Silué, 2009).

Sclerotinia stem rot (SSR), caused by *Sclerotinia sclerotiorum,* is a fungal disease that can cause considerable yield losses, with up to 70% infection incidence in winter oilseed rape when the

conditions are suitable (Koch *et al.*, 2007). Resistance for this disease has been identified in *B. oleracea* (Mei *et al.*, 2011, 2013) and *B. napus* (Taylor *et al.*, 2015), contrary to the high susceptibility found in *B. juncea* (Li *et al.*, 2009). Recently, *Sclerotinia* resistance governed by several loci found in a wild C-genome species (*B. incana*) was introduced into *B. napus* via an interspecific hexaploidy hybrid bridge method (Mei *et al.*, 2015, 2020). Through pyramiding three major QTLs, the BC_1F_8 line gained approximately 35% resistance when compared to the *B. napus* parent (Mei *et al.*, 2020). Another strong source of resistance to SSR was found in *Brassica fruticulosa* (Rana *et al.*, 2017). Subsequently, this resistance was transferred into a susceptible *B. juncea* genotype, producing introgressed lines with increased resistance, with a reduced lesion size of up to 69%. From the introgressed material it was also possible to select euploid and high pollen fertility lines, making it an excellent source to be utilized in future breeding programs (Rana *et al.*, 2017).

Blackleg or phoma stem canker (caused by *Leptosphaeria maculans*) mainly affects rapeseed grown in Canada, Europe and Australia (West *et al.*, 2001). One of the ways to control this disease is by sowing resistant cultivars, hence the need to find new genetic resources is always an ongoing process. Resistance to blackleg was found in lines of *B. napus* (Delourme *et al.*, 2006; Rimmer, 2006; Light *et al.*, 2011) and *B. rapa* subsp. *sylvestris* (Yu *et al.*, 2005, 2008). To date, no resistance R gene against blackleg has been observed on the C *Brassica* genome, although some possible *in-silico* candidates have recently been proposed (Ferdous *et al.*, 2020). Other kind of resistances that involve more than just one gene are known as quantitative disease resistance. This resistance is associated to particular genomic region/s or a quantitative trait loci that contributes to a partial level of disease resistance, usually more complex to identify due to its nature but in the long term, harder for the pathogen to overcome(Pilet-Nayel *et al.*, 2017). Several blackleg resistance QTL have been identified in spring-type *Brassica napus* (Larkan *et al.*, 2016) and in diversity set of *Brassica napus* (Jestin *et al.*, 2011; Rahman *et al.*, 2016; Raman *et al.*, 2016).

Brassica oleracea is the major host for black rot (*Xanthomonas campestris* pv. *campestris*) (Vicente *et al.*, 2001). This disease can cause severe damage, affecting up to 50% of the crop (Singh *et al.*, 2011). Several resistant lines have been found in *B. oleracea* (Lema *et al.*, 2012; Saha *et al.*, 2016; Ribeiro da Silva *et al.*, 2020) and *B. rapa* (Lema *et al.*, 2015). In search of resistance in other subgenomes than the C, a single gene resistance locus was identified in *B.*

carinata, located on linkage group B7 (Sharma *et al.*, 2016). Later on, this resistance was introgressed into *B. oleracea* using embryo rescue (Sharma *et al.*, 2017).

Resistance to white rust (WR) caused by the pathogen *Albugo candida* has been found in *B. juncea, B. napus, B. rapa* and *B. carinata* varieties (Panjabi-Massand *et al.*, 2010; Awasthi *et al.*, 2012). Quite recently, a *B. juncea* Chinese vegetable type mustard called Tumida was found to be resistant to WR, for which a responsible locus was located on linkage group A06 (Bhayana *et al.*, 2020). Different *Brassica* genotypes and allies from diverse origins were tested against *Pseudocercosporella capsellae* (white leaf spot disease) in field and/or controlled conditions, and genotypes from *B. carinata, B. juncea, B. napus, B. oleracea* and *B. fruticulosa* shown to be highly resistant (Gunasinghe *et al.*, 2014, 2017). By comparing resistant and susceptible lines derived from three allotetraploid *Brassica* types, Gunasinghe et al. (2016) identified a resistant *B*. *carinata* line possessing stomata prone to closure to inhibit pathogen penetration. Also, a higher stomata density was observed in the susceptible lines.

2.4.3 Abiotic stress tolerances

Abiotic stress tolerances also vary across the *Brassica* species. Salt tolerance has been shown to be greater in the allopolyploids *B. juncea*, *B. napus* and *B. carinata* than in their diploid parents (Ashraf *et al.*, 2001). Similar effects were observed when comparing salinity tolerance between various *Brassica* genotypes and ploidies (Kumar *et al.*, 2009). In a different study, where tetraploid turnips (*B. rapa*) were compared to diploid progenitors, it was also shown that this increase in ploidy positively affects salinity tolerance (Meng *et al.*, 2011). A wide diversity set of *B. napus* accessions (85 inbred lines) were tested for salt tolerance under hydroponic conditions (Yong *et al.*, 2015). The results showed significant variation in shoot fresh weight and dry weight between the different accessions and, at the same time, there was no correlation between sodium ion accumulation in leaves and the salt tolerance index.

Screening of nine different *B. juncea* genotypes resulted in the discovery of one tolerant genotype (Varuna) among them (Hayat *et al.*, 2011). In *B. juncea,* several other tolerances have been observed, such as heat stress (Wilson *et al.*, 2014) and cadmium tolerance (Gill *et al.*,

2011; Irfan *et al.*, 2014). Nevertheless, many of the results obtained for tolerance to heavy metals depend on the methods utilized to screen the tolerance (Hernández-Allica *et al.*, 2008) and also not all of them are easily comparable due to these differences (reviewed in (Mourato *et al.*, 2015)).

Polluted soil, water or air can be of great danger to human health. Fortunately, we can use plants to remove those contaminants, a term known as phytoremediation (reviewed in (Salt *et al.*, 1998)). An excellent example of this is *B. juncea* var. *foliosa*, which has the potential to be used in phytoremediation in thorium (Th) contaminated soils due to its ability to tolerate this metal (Zhou *et al.*, 2016a). Under low concentrations of Th, *B. juncea* var. *foliosa* grew better, but under high concentrations plant metabolism and growth rates were affected. Some of the *Brassica* vegetable types, like *Brassica rapa* subsp. *pekinensis* (Chinese cabbage), also have the capacity to accumulate high amounts of heavy metals without any obvious symptoms, presenting a potential risk for human food contamination (Xiong & Wang, 2005).

Lack of water during flowering can heavily impact the final yield production of plants. Thankfully, we can use the available germplasm of a species to investigate how well they are able to cope, and even recover if they were submitted to water stress. Phenotyping for drought stress tolerance in *B. napus* under simulated normal and osmotic stress conditions in a hydroponic system combined with GWAS revealed 16 water stress-tolerant accessions and 16 SNP loci associated with osmotic stress response (Zhang *et al.*, 2015c). When comparing single genotypes of *B. napus*, *B. rapa* and *B. juncea* under simulated drought stress (using PEG-6000) it was found that *B. juncea* was more drought tolerant than the other two species (Alam *et al.*, 2014). A study of drought tolerance in *B. napus* pre- and post-flowering conditions found 3 and 4 different accessions tolerant to drought, respectively (Zhu *et al.*, 2011). A closer characterization of drought tolerance mechanisms in *B. napus* revealed that individual strategies vary strongly between accessions, but common drought tolerance genes might exist (Schiessl *et al.*, 2020).

2.4.4 Other traits of agronomic interest

A number of other miscellaneous traits of agronomic importance are also present in various *Brassica* species. Cytoplasmic male sterility (CMS) is a widely utilized system to produce F_1 hybrids in *Brassica* crops taking advantage of the high hybrid vigour observed in seed yield (Yamagishi & Bhat, 2014). Several systems have been found in species like *B. juncea* (hau CMS, (Wan *et al.*, 2008))*, B. napus* (nap and pol CMS, (Brown, 1999))*, B. rapa* (YSMS-6 (Bhajan, 2000); eru CMS (Peng *et al.*, 2015)), *Raphanus sativus* (ogu CMS (Ogura, 1968)), *B. oleracea* (Zhiyuan *et al.*, 1995; Fang *et al.*, 1997) and a system produced by the cross between *B. napus* and *B. carinata* (NCa (Wei *et al.*, 2009)).

Leaves are very important organs, where process like photosynthesis, respiration and transpiration take place, and also are the initial barrier against environmental conditions. Leaf composition can also act as a barrier against herbivore attack (Žnidarčič *et al.*, 2008; Bohinc *et al.*, 2014). In *B. juncea* leaf morphology was studied in 10 wild accessions (Huangfu *et al.*, 2009): the different populations varied in leaf thickness, wax content, and leaf surface, among other morphological traits. Interestingly, some of the phenotypes analyzed also correlated with herbicide (glyphosate) resistance in the populations, especially leaf thickness, with an R^2 of 0.72.

Pod shattering, from an evolutionary point of view, is a great mechanism for seed dispersal. Unfortunately, from an economical point of view, in *Brassica* oilseed crops can cause great seed losses during harvesting, which under normal conditions can reach up to 2-5%, and when the conditions are less than optimal values over 20% or up to 50% can be obtained (Price *et al.*, 1996). Pod shatter resistance is present naturally in *B. carinata*, *B. juncea* and *B. rapa* genotypes (Raman *et al.*, 2014). On the other hand, the variation present in *B. napus* for pod shattering is more limited. For example, in a study where 229 *B. napus* accessions were investigated for silique shattering resistance, just two varieties were fully resistant (Wen, 2008).

Novel traits may also be utilised to produce niche *Brassica* types for different purposes. For instance, *Brassica* species are characterized by the ubiquitous presence of glucosinolates, although the amount and composition of these compounds varies depending on the tissue or cultivar analyzed (Verkerk *et al.*, 2010; Sun *et al.*, 2019). Glucosinolates are secondary metabolites that have been associated in plants with insect resistance (Evivie *et al.*, 2019), fungal resistance (Bednarek *et al.*, 2009; Buxdorf *et al.*, 2013), signalling molecules in the auxin

pathway (Katz *et al.*, 2015), its involvement in other biological processes like flowering time and stomatal closure (reviewed in (Barco & Clay, 2019)) and even the possible contribution in preventing certain types of human cancer like lung, stomach and prostate when included in the diet (reviewed in (Traka & Mithen, 2009)). Most commercial oilseed *Brassica* cultivars have been bred to contain low levels of glucosinolates, which is more desirable for edible oil. However, there is a niche for specific glucosinolate profiles that are desirable for other applications, for example in industrial oil production (Princen, 1979).

Carotenoid content is another trait of interest that can potentially be manipulated and bred to produce edible plants with specific profiles, to fit human needs. In *B. oleracea,* diverse carotenoid composition was observed in a set of 30 different cultivars from various origins (Mageney *et al.*, 2016). In *B. rapa* spp. *pekinensis*, a hybrid produced by the cross of two incompatible cultivars produces a hybrid with orange inner leaves (Yangjun *et al.*, 2005). Interestingly, also in *B. rapa* cultivars, the production of other pigments (anthocyanin) has been associated with cold and freezing resistance (Ahmed *et al.*, 2015).

2.5 Hybridization for crop improvement in *Brassica*

Genetic diversity within and between species is a prerequisite for breeding and crop improvement. In order to improve yields, increase disease resistance and refine oil qualities to cater to various nutritional and industrial purposes, it is imperative to introduce new sources of genetic diversity into existing elite cultivars (Allender & King, 2010). In the Brassicaceae, new variation can be generated by hybridization involving adapted cultivars, wild types and landraces or exotic germplasm such as different species (Friedt *et al.*, 2018).

Interspecific hybridization is useful in the introgression of desirable traits from one species to another and there are different approaches for transferring traits through interspecific hybridization (Prakash et al. 2009; Mason and Chèvre 2016). The success of crosses between any two parents can be determined by observing their pollen germination, pollen tube growth, embryo development, and seed set (Bhat & Sarla, 2004). Hybrid incompatibilities occur when hybrids are sterile, less fit or even non-viable compared to their progenitors: this serves as a

reproductive isolation barrier which can lead to speciation (Coyne & Orr, 2004). Within the *Brassica* genus, incompatibilities may occur between different species, cultivars or species with different ploidy levels (Nishiyama *et al.*, 1991a; Fitzjohn *et al.*, 2007). To date, a number of genes with diverse functions, including those involved in oxidative respiration, nuclear trafficking, DNA-binding, and plant defence have been linked to hybrid incompatibilities (Johnson, 2010; Rieseberg & Blackman, 2010), but the underlying genetic and molecular mechanisms are not yet fully understood (Vaid & Laitinen, 2019).

Other mechanisms that prompt hybrid incompatibility include conflicts resulting from the unequal parental contribution to the formation of hybrid or developing seed (Carputo *et al.*, 2003; Johnson, 2010; Köhler *et al.*, 2010). This is often seen in the different phenotypes or success rate obtained when reciprocal crosses are made. In crosses between *Brassica* species, the choice of maternal species has a big effect on the success of the cross (Fitzjohn *et al.*, 2007; Chen *et al.*, 2011b). Another well-known example is the cytoplasmic male sterility (CMS) which is a maternally inherited trait characterized by the inability of a plant to produce functional pollen (Eckardt, 2006). Hybrid chlorophyll deficiency causes white-coloured cotyledons and this has been identified to occur as a result of incompatibility between the plastid genome and the nuclear genome (Ureshino *et al.*, 1999; Okamoto & Ureshino, 2015).

Hybrid necrosis or death of young seedlings is another form of post-zygotic incompatibility which is associated with complexities in gene interaction (Potts & Dungey, 2004; Okamoto & Ureshino, 2015). In Arabidopsis for instance, it has been revealed that conflict between two gene variants or loci *(DANGEROUS MIX 1* (*DM1*) and *DANGEROUS MIX 2* (*DM2*)) may trigger defence reactions which can be detected phenotypically in hybrids as necrotic lesions on leaves and a decline in growth and fertility (Bomblies & Weigel, 2007; Chae *et al.*, 2014). *ACCELERATED CELL DEATH 6* (*ACD6*) is another gene that causes hybrid necrosis when its allele variants interact leading to the activation of pathogen-recognition receptors and trigger autoimmune response to pathogens in first generation hybrids of *A. thaliana* (Todesco *et al.*, 2014; Tateda *et al.*, 2015; Świadek *et al.*, 2017).

Even after successful pollen germination and fertilization, the abnormal growth of the endosperm can interfere with normal seed development (Haig & Westoby, 1991; Lafon-Placette & Köhler, 2016). Similarly, in *Brassica* species, interspecific hybridization does not always lead to the production of mature seeds, as a result of irregularities in endosperm

development (Nishiyama *et al.*, 1991a). Failure of endosperm development in hybrids may occur as a result of unbalanced parental genome dosages or genomic imprinting (Köhler *et al.*, 2010).

2.6 Transferring useful traits from wild relatives to crop species: how does it work?

Although a major QTL *PrBn* (for pairing regulator in *B. napus*) and other minor QTL have been observed to affect non-homologous chromosome pairing frequencies in *Brassica napus* allohaploids (Jenczewski *et al.*, 2003; Liu *et al.*, 2006), *Brassica* species generally have weak, quantitative regulation of meiosis, which readily permits hybridization and introgressions to transfer useful traits between genomes (Figure 1).

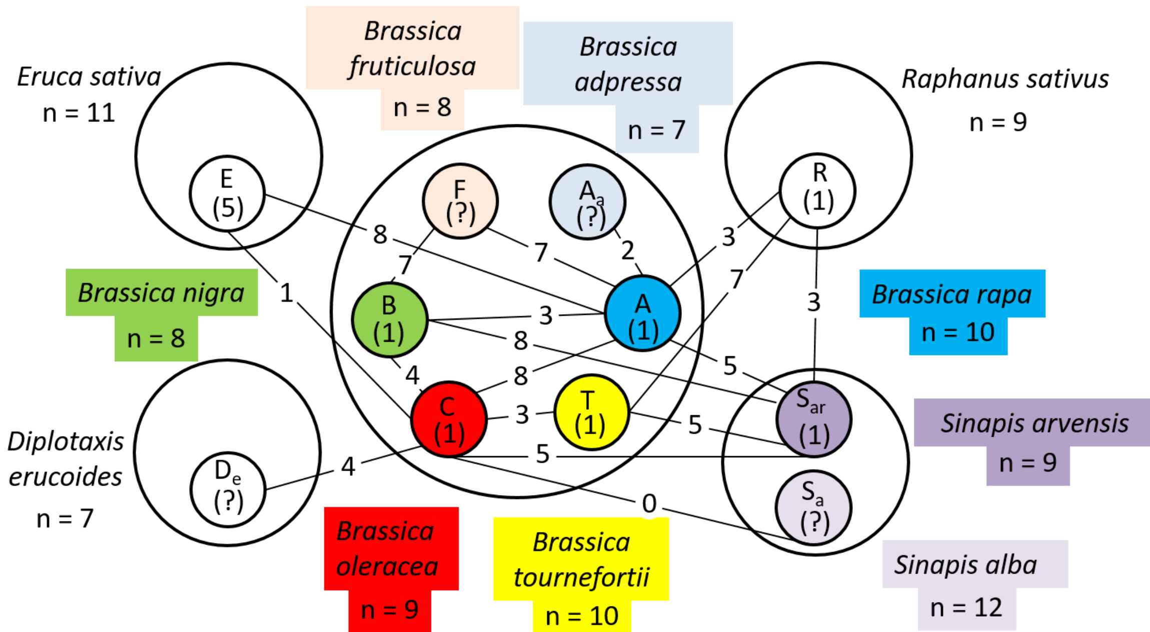


Figure 1 (adapted from (Mizushima, 1980)**): Genome interrelationships in *Brassica* and allied genera.** Numbers in brackets represent the number of autosyndetic bivalents observed in haploids, while numbers on lines indicate the maximum number of bivalents observed in interspecific hybrids between the two species (necessary for transferring traits between genomes).

The first step in transferring useful traits from wild relatives to crops is to identify which wild relative germplasm carries the trait of interest, and preferably also the genetic basis for this trait. Ideally, the target germplasm will be within the same species, and the trait will be carried by a single major gene locus. Unfortunately, this situation is rarely found. Firstly, many species are relatively inbred, lacking the genetic and trait diversity necessary for further specific improvements. In the *Brassica* genus, this is particularly true in major crop species *B. napus* (rapeseed), for which no "wild" forms exist (Dixon, 2007), and in which (for example) little to no resistance to insect predation is thought to exist (Hervé, 2018). Hence, it is often necessary to look outside this so-called "primary" germplasm pool for traits. Secondly, although some traits are often carried by major genes, such as resistance to blackleg/Phoma disease (Rimmer, 2006; Leflon *et al.*, 2007) or resistance to clubroot (Manzanares-Dauleux *et al.*, 2000), most traits, including drought tolerance (Fletcher *et al.*, 2015, 2016; Zhu *et al.*, 2016), flowering time (Schiessl *et al.*, 2014, 2015), and of course yield (Zhou *et al.*, 2014; Luo *et al.*, 2017), tend to be the product of multiple genes, genetic factors or gene networks.

The reason that it is better to have traits which are a) present in closely-related species and b) controlled by a single locus is because of the mechanisms by which we transfer traits from the wild to crop germplasm. The physical transfer of genetic material between two germplasm groups usually needs to occur via one or more crossovers between chromosomes in the hybrid which has been produced between them, which (usually) has 50% genetic material from each parent (Figure 2).

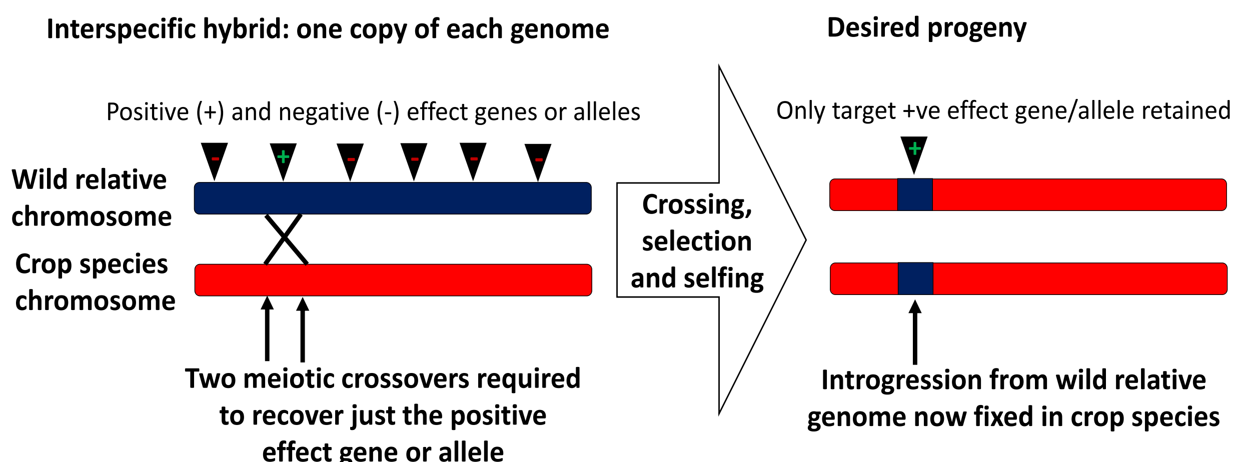


Figure 2: Meiotic crossovers in the interspecific hybrid are required between chromosomes belonging to the wild relative and the crop species (homoeologous crossovers) for production of introgression lines.

While it is relatively easy to make hybrids within a species by hand-emasculation and pollination, this becomes much more difficult with increasing genetic distance between the wild relative and the crop (Fitzjohn *et al.*, 2007). As well, the subsequent chance of recovering recombination events is greatly reduced if there is little relationship between the two sets of chromosomes present in the hybrid, such that they rarely pair and recombine with each other (Mason & Chèvre, 2016)(Figure 1). If multiple genetic loci need to be transferred, even more crossovers need to form, and this further reduces the chance of recovering the desirable trait in segregating hybrid progeny (Mason & Chèvre, 2016). Depending on the genomic location of the locus of interest, it may not even be possible to produce recombinants through conventional means, as crossovers are not evenly distributed across chromosomes (reviewed by (Choi & Henderson, 2015)), and are actively suppressed in others, such as centromeres (Talbert & Henikoff, 2010). Hence, some genomic regions are very unlikely to recombine during meiosis: when considering two target regions in a hybrid, the probability of a natural crossover forming between these two may be so low as to be effectively non-existent. Also, every transfer has the potential to introgress large blocks of undesirable genetic variation as well as the desirable genetic variation conferring the trait of interest (linkage drag), as normally, a large chromosomal segment will be introgressed from a single crossover. In the case of intraspecific crosses or crosses between species with very high genomic similarity this is not such a big problem: subsequent recombination events may occur through backcrossing to the crop parent, and thus reduce the size of the introgression block (Figure 2). This eliminates undesirable genetic variation while retaining the locus of interest. However, further recombination events cannot be guaranteed in the case of wide crosses, which may mean that the resulting introgression region is large and carries a high number of undesirable genetic variants. This problem was classically encountered in *Brassica* breeding with the production of the restorer lines for the “Ogura” CMS system developed from radish wide hybrids (Pellan-Delourme & Renard, 1988), and for which gamma ray induction of chromosome breakage was required to reduce introgression size (Primard-Brisset *et al.*, 2005).

2.7 Improving our chances of recovering introgressions of useful traits from relatives into crops to build climate resilience

So, what can we do to facilitate transfer of genetic loci and traits of interest from wild relatives into crops to build climate resilience? Good experimental planning and prior knowledge is key to improving success rates. Although in some cases very little is known about a) genetic control of the target trait or phenotype in question, b) ease of hybrid production, c) frequency and distribution of crossovers in the interspecific hybrid or d) chance of recovering successful introgressions, most of the time at least some of this information should already be known, and can be used to predict the amount of time and effort likely required to achieve this goal. Recent developments in genomics and bioinformatics techniques are predicted to help a lot in this respect (for review see (Zhang & Batley, 2020)). However, there are also a number of specific methods or considerations that can be used to facilitate this process.

Hybrid generation is not always successful especially across different ploidy levels. However, a number of crossing approaches can be used to facilitate trait transfer through interspecific hybridization in *Brassica* species and their relatives (Prakash et al. 2009; Mason and Chèvre 2016). Hybridisation is generally more successful between species which share a genome e.g. between a tetraploid and a diploid progenitor species, or between two tetraploids which share a progenitor ((Prakash *et al.*, 2009); reviewed in Mason and Chèvre 2016). Hybridization between diploids and tetraploids that do not share a genome is also possible, and the resulting tri-genomic hybrids can be used as a bridge to introgress genetic diversity between species in further hybridization events, or can be induced by colchicine doubling to generate allohexaploids (Chen *et al.*, 2011b). While interspecific hybridization is very useful for hybrid speciation and crop improvement in the *Brassica* genus, hybridizations can also be made between genera. Hybridization involving the *Brassica* crop species is often successful using only hand-pollination methods (Fitzjohn *et al.*, 2007). However, hybridization can be facilitated by tissue culture techniques which “rescue” fertilized ovules or embryos before these are aborted by the maternal parent (reviewed by (Sharma *et al.*, 1996)). For wider crosses somatic fusion may also be possible, where somatic cells (usually protoplasts) are directly induced to combine in tissue culture (reviewed by (Navrátilová, 2004)), although this method frequently results in aneuploidy (loss or gain of individual chromosomes from a set)(Gaebelein & Mason, 2018). A

common example is the protoplast fusion of rapeseed and radish. This method was employed in generating the Ogura cytoplasmic male sterile *B. napus* system and was very beneficial in attaining double low restorer lines with a decrease in erucic acid and glucosinolate content (Pelletier *et al.*, 1983; Primard-Brisset *et al.*, 2005). Better success may also be achieved in some cases by chromosome doubling the parent species before hybridization is attempted (Frandsen, 1947; Heyn, 1977; Akbar, 1990); this can also be achieved by various chemical treatments and methods (reviewed by (Dhooghe *et al.*, 2011)). Other approaches used in tackling these incompatibilities include hot water treatments against pre-fertilization barriers (Prabha *et al.*, 1982), early pollination of stigmas or stump pollination, *in vitro* pollination (Reviewed in Katche et al. 2019), and artificially supplied nutrients and hormones against post-fertilization barriers (Sharma *et al.*, 1996; Abel *et al.*, 2005; Prakash *et al.*, 2009).

Once a hybrid is produced, every meiosis in this hybrid (every pollen or ovule produced) has the potential to produce recombinant chromosomes (introgressions) between the source and target genomes. Meiotic recombination is an important aspect of breeding, as it ensures plant fertility and the generation of diversity through shuffling of genetic information. However, CO localization is uneven across the genome, with 80% of all COs occurring in about 25% of genomic regions in most plants (usually at the distal euchromatic regions) (Darrier et al. 2017). Obtaining the meiotic recombination required for crop improvement is also challenging in plants with low CO frequencies. Knowledge of how often recombination events occur in different types of hybrid is invaluable in knowing approximately how many progenies may need to be obtained in order to recover the desired introgression. Even better, knowledge about genome-wide distribution of recombination rates in hybrids of different types would allow predictions of success in introgression of particular genetic loci before experiments even start. However, COs can potentially be increased through several mechanisms including the knockout of anti-crossover regulators such as FANCM, RECQ4 and FIGL1, or combining the knockout of anti-CO regulators with an increase in the dosage of ZMM protein HEI10, and through mutagenesis approaches (Blary and Jenczewski 2019). In *Brassica* allotriploid AAC hybrids, there is an increase in CO rates between the homologous A chromosomes (Leflon et al. 2010); better understanding and characterisation of this effect may be helpful in applying this crossover boost to other hybrid types. In future, it may also be possible to manipulate the regulation of chromosome pairing between genomes in order to boost the frequency of crossovers and change their genomic locations (reviewed by (Blary & Jenczewski, 2019)).

To facilitate introgressions in the absence of other information about recombination frequencies or crossover distributions, target genes should ideally be close to chromosome telomeres and in chromosomal regions with high homoeology (or homology if possible) between the two sets of chromosomes in the hybrid. In *B. juncea* × *B. carinata* BBAC hybrids, A-C pairing is extraordinarily frequent, with an average of 7 A-C allosyndetic pairs per meiosis (Mason *et al.*, 2010), almost always between primary homoeologous regions (Mason *et al.*, 2014a). In the same hybrids, autosyndetic recombination events (A-A and C-C in the haploid genomes) occur at a rate of approximately one event per 2 meioses (Mason *et al.*, 2010), but likely only between the largest blocks resulting from the ancestral genome triplication, involving up to a chromosome arm (Mason *et al.*, 2014a). In hybrids resulting from the cross *B. napus* × *B. nigra*, B-A/C allosyndesis is observed in 1/3 meioses in ABC triploid hybrids, and 1/6 meioses in AABBCC allohexaploid hybrids (Gaebelein *et al.*, 2019a), although which genomic regions are recombining is not known. Although hypothetically any genomic similarity can trigger recombination, crossovers have strong "preferences", and will form between whatever chromosomes are present on order of sequence similarity first (Grandont *et al.*, 2014). In the absence of homologous pairing partners, recombination will occur most frequently between the most closely-related (or possibly largest) homoeologous regions (Nicolas *et al.*, 2007, 2009; Mason *et al.*, 2014a). Regions of primary and secondary (resulting from ancestral triplication) homoeology have been well-defined for quite some time for the A and C genomes (Parkin *et al.*, 2003; Schranz *et al.*, 2006; Cheng *et al.*, 2013), and to a lesser extent the B genome (Lagercrantz & Lydiate, 1996). Now, with the availability of reference genome sequences, these relationships have been even better elucidated (The Brassica rapa Genome Sequencing Project Consortium, 2011; Chalhoub *et al.*, 2014; Parkin *et al.*, 2014; Yang *et al.*, 2016b; Wang *et al.*, 2019), and subsequently can be used to predict the probable locations of genomic introgressions, even though we are still lacking a lot of information about where crossovers are most likely to form.

Conventionally, we can boost our chances of transferring desirable loci by selecting good targets: single, major gene effects, present in close relatives to our crop of interest. However, several agronomic traits of interest, including yield, plant height and flowering time, are controlled by many genes and heavily influenced by the environment, and thus present a greater challenge. The genetic control or mode of inheritance of a desired trait can be examined through marker analysis or by observing how traits segregate in progenies. Owing to the recent

advancement in genotyping, gene editing and marker technologies, the characterization and introgression of our gene of interest, genomic regions (complex traits) or even pyramiding of multiple QTLs can readily be done. In a number of studies, introgression was successfully achieved via a combination of different hybridization schemes. In general, creation of a suitable mapping population to elucidate the genetic control of the trait, followed by association of phenotypes with genotypes to identify genomic regions of interest and subsequent development of marker assisted selection (MAS), is an excellent strategy to facilitate production of introgression lines. For instance, to introgress *Sclerotinia* resistance into rapeseed, Mei et al. (2020) transferred multiple resistant loci from wild *B. oleracea* through backcrossing, selfing and MAS. A different approach was used by Mei et al. (2015) to introgress *Sclerotinia* resistance from *B. incana* (a wild relative of *Brassica oleracea*) *into B. napus* bridged by a hexaploidy step.

Tracking of introgressions can be sped up by marker-assisted selection, or possibly even by genomic selection in the case of multi-locus traits being transferred between close relatives. Marker-assisted selection has been effectively used to track B-genome introgressions related to *Sclerotinia* disease resistance in *Brassica napus* (Navabi *et al.*, 2010), to produce higher-quality restorer lines carrying the *Rfo* restorer gene for the *Ogura* CMS system in *B. juncea* by reducing the size of the radish introgression (Tian *et al.*, 2014), and to map and move clubroot resistance gene *Rpb1* from *B. rapa* into *B. napus* (Chu *et al.*, 2013). Genome-wide marker assisted-selection has also been used in several studies to recover subgenome-substitution or resynthesised lines. This approach has been used to produce "new-type" *B. napus* (AACC) with an A genome from *B. rapa* (A^r) and a C genome (C^c) from *B. carinata* (Xiao *et al.*, 2010), through selection for A^r and C^c alleles over A^n and C^n, as well as to extract the *B. napus* A genome by eliminating the C genome (Pelé *et al.*, 2017b). Hence, genome-wide marker-assisted selection may be worth considering in the case of complex traits which are being moved between species which share a subgenome (e.g. species within the Triangle of U).

2.8 Conclusions

In this review, we introduce potential impacts of climate change on crop production and the Brassicaceae crops, provide a reference for useful traits present in each of the *Brassica* "Triangle of U" species and then offer concrete advice for structuring and optimising introgression breeding programs. Success in transferring agronomically relevant traits between species depends on factors such as similarity between the source (e.g. wild relative) and target (e.g. crop) genomes, the ease of hybrid production, the frequency and distribution of crossovers in the interspecific hybrid meiosis, and subsequently the ease of recovery of introgression lines. Regardless of the not considerable difficulties involved in the use of wild relatives for crop improvement, this method offers a great deal of as-yet unexplored potential for the improvement of *Brassica* crops, and in improving crop resilience and resistances in the face of global climate change.

3. Allele segregation analysis of F_1 hybrids between independent *Brassica* allohexaploid lineages.

3.1 Abstract

In the *Brassica* genus, we find both diploid species (one genome) and allotetraploid species (two different genomes) but no naturally occurring hexaploid species (three different genomes, AABBCC). Although hexaploids can be produced via human intervention, these neo-polyploids have quite unstable genomes and usually suffer from severe genome reshuffling. Whether these genome rearrangements continue in later generations and whether genomic arrangements follow similar, reproducible patterns between different lineages is still unknown. We crossed *Brassica* hexaploids resulting from different species combinations to produce five F_1 hybrids and analyzed the karyotypes of the parents and the F_1 hybrids, as well as allele segregation in a resulting test-cross population via molecular karyotyping using SNP array genotyping. Although some genomic regions were found to be more likely to be duplicated, deleted, or rearranged, a consensus pattern was not shared between genotypes. *Brassica* hexaploids had a high tolerance for fixed structural rearrangements, but which rearrangements occur and become fixed over many generations does not seem to show either strong reproducibility or to indicate selection for stability. On average, we observed 10 de novo chromosome rearrangements contributed almost equally from both parents to the F_1 hybrids. At the same time, the F_1 hybrid meiosis produced on average 8.6 new rearrangements. Hence, the increased heterozygosity in the F_1 hybrid did not significantly improve genome stability in our hexaploid hybrids and might have had the opposite effect. However, hybridization between lineages was readily achieved and may be exploited for future genetics and breeding purposes.

3.2 Introduction

The *Brassica* genus belongs to the Brassicaceae family (Warwick *et al.*, 2006) and includes many economically valuable crop species. The *Brassica* crops can be broadly classified into vegetable, oilseed, fodder and condiment types. During the 1930s, the chromosome number and genetic relationships between the cultivated *Brassica* species were established in what we know as the triangle of U (U, 1935): the diploid species *B. rapa* (AA, n = 10), *B. nigra* (BB, n = 8) and *B. oleracea* (CC, n = 9) were defined as progenitors of the allotetraploid species *B. juncea* (AABB, *n* = 18), *B. napus* (AACC, *n* = 19), and *B. carinata* (BBCC, *n* = 17), which originated via pairwise spontaneous hybridization between these diploids. The *Brassica* vegetables, *B. oleracea* and *B. rapa,* are characterized by vast diversity in subspecies and varieties (Cheng *et al.*, 2016). In these species, it is possible to find different domesticated morphotypes distinguishable by leaf types, inflorescence types or enlargement of roots or stems. In *Brassica* species it is also possible to find traits of agronomic interest, such as disease resistance (Chevre *et al.*, 1996; Mei *et al.*, 2011, 2013; Peng *et al.*, 2014; Taylor *et al.*, 2015; Fredua-Agyeman *et al.*, 2019), abiotic stress tolerance (Gill *et al.*, 2011; Hayat *et al.*, 2011; Wilson *et al.*, 2014; Irfan *et al.*, 2014) and pod shatter resistance (Zhang *et al.*, 2016)*,* among other potential traits (reviewed in (Katche *et al.*, 2019)).

Commonly, in the *Brassica* genus we find both diploid and tetraploid species but no naturally occurring hexaploid species (AABBCC = 2n = 6x = 54). Despite this, it is possible to synthesize this hybrid via human intervention. The three most common cross combinations to produce an allohexaploid are: (i) *B. carinata* × *B. rapa* (Fig. 1a)*,* (ii) *B. juncea* × *B. oleracea,* and (iii) *B. napus* × *B. nigra* (reviewed in (Gaebelein & Mason, 2018)), that from now on will be referred to as "carirapa", "junleracea", and "naponigra" allohexaploid types (Zou *et al.*, 2010). All of these hybrids are usually colchicine-treated to induce chromosome doubling following hybridization between a diploid and a tetraploid species. A more recent method to produce an allohexaploid is via two-step crossing and relies on unreduced gamete production: the hybridization between *B. napus* × *B. carinata* × *B. juncea* (Mason *et al.*, 2012), referred to as "NCJ" allohexaploid types (Fig. 1a). Until now, many of the allohexaploid genotypes produced were solely used to cross to *B. napus,* either to introgress genetic diversity (Li *et al.*, 2004, 2006; Jiang *et al.*, 2007; Zou *et al.*, 2010, 2018; Hu *et al.*, 2019), or to transfer specific traits such as yellow seededness (Meng *et al.*, 1998; Rahman, 2001) or fungal disease resistance (Sjödin & Glimelius, 1989). Despite this,

a new allohexaploid hybrid has the potential to become a new species with the advantage of combining all the different traits present in the six U´s triangle species, thus broadening the genetic resources available for breeders (Chen *et al.*, 2011a). In such an allohexaploid, it would also be possible to take advantage of "fixed heterosis" (Abel *et al.*, 2005), where the heterosis present between the subgenomes (A, B and C) can be maintained in inbreeding lines. In addition, new phenotypes which are not present in the original parents may develop from the different crosses via novel mutations due to the hybridization event (Udall & Wendel, 2006; Kaur *et al.*, 2014).

Allohexaploid *Brassica* (AABBCC = 2n = 54), as a new polyploid has to overcome the major challenge of establishing regular meiosis (Pelé *et al.*, 2018). The correct pairing of homologous chromosomes during meiosis is a key factor to ensure correct cross-over and chromosome segregation. In the case of this allohexaploid, meiosis is challenging due to the presence of three ancestrally homologous chromosome sets from different evolutionary lineages, known as homoeologous chromosomes (Ramsey and Schemske 2002). If non-homologous pairing during meiosis occurs, it can lead to different chromosomal rearrangements, such as deletions, duplications and translocations (Udall *et al.*, 2005), heavily affecting genome stability. In a synthetic trigenomic hybrid, we usually observe the A and the C genome are more likely to pair during meiosis, compared to the A-B or C-B homoeologs (Mason *et al.*, 2010). In the case of natural allopolyploids, such a *B. napus,* meiosis progresses normally, although non-homologous exchanges do still occur at lower frequency (Parkin *et al.*, 1995; Udall *et al.*, 2005; Higgins *et al.*, 2018).

Many different types of cross-combinations have been tested to produce meiotically stable 2n = AABBCC allohexaploids (reviewed in (Gaebelein & Mason, 2018)). It has been shown that early generation (F_2) carirapa hexaploids have low pollen viability and irregular configurations during meiosis (Howard, 1942; Iwasa, 1964). It has also been seen that fertility and number of 2n = 54 (putatively euploid) plants can increase by selection over successive generations (Tian *et al.*, 2010; Zhou *et al.*, 2016b). Genotype combinations seem to play a great role in the success of the hexaploid, as many of the more stable and fertile hexaploids produced are derived from just a few lines (Tian *et al.*, 2010). A specific *B. rapa* genotype (R01) was also reported to result in a meiotically stable carirapa allohexaploid (Gupta *et al.*, 2016). Carirapa combinations that included this *B. rapa* genotype had high frequency of bivalent formation during meiosis,

progeny with the expected 54 chromosomes, and no major translocations observed (Gupta *et al.*, 2016). Similarly, genotype-specific effects on fertility and meiotic stability have been observed in NCJ hexaploids (Mwathi *et al.*, 2017).

In the present study we analyzed chromosome and allele segregation resulting from the meiosis of five different F_1 hybrids produced between crosses of putatively stable *Brassica* hexaploids carirapa and NCJ. We hypothesized that the new hybrids might show improved meiotic stability (more regular chromosome segregation and fewer non-homologous recombination events) compared to their parents. We also aimed to determine if chromosome rearrangements found at high frequency or in independent lineages of the parent hexaploid types (e.g. both in NCJ and carirapa) might be associated with improved meiotic stability, since all lineages underwent strong selective pressure for fertility.

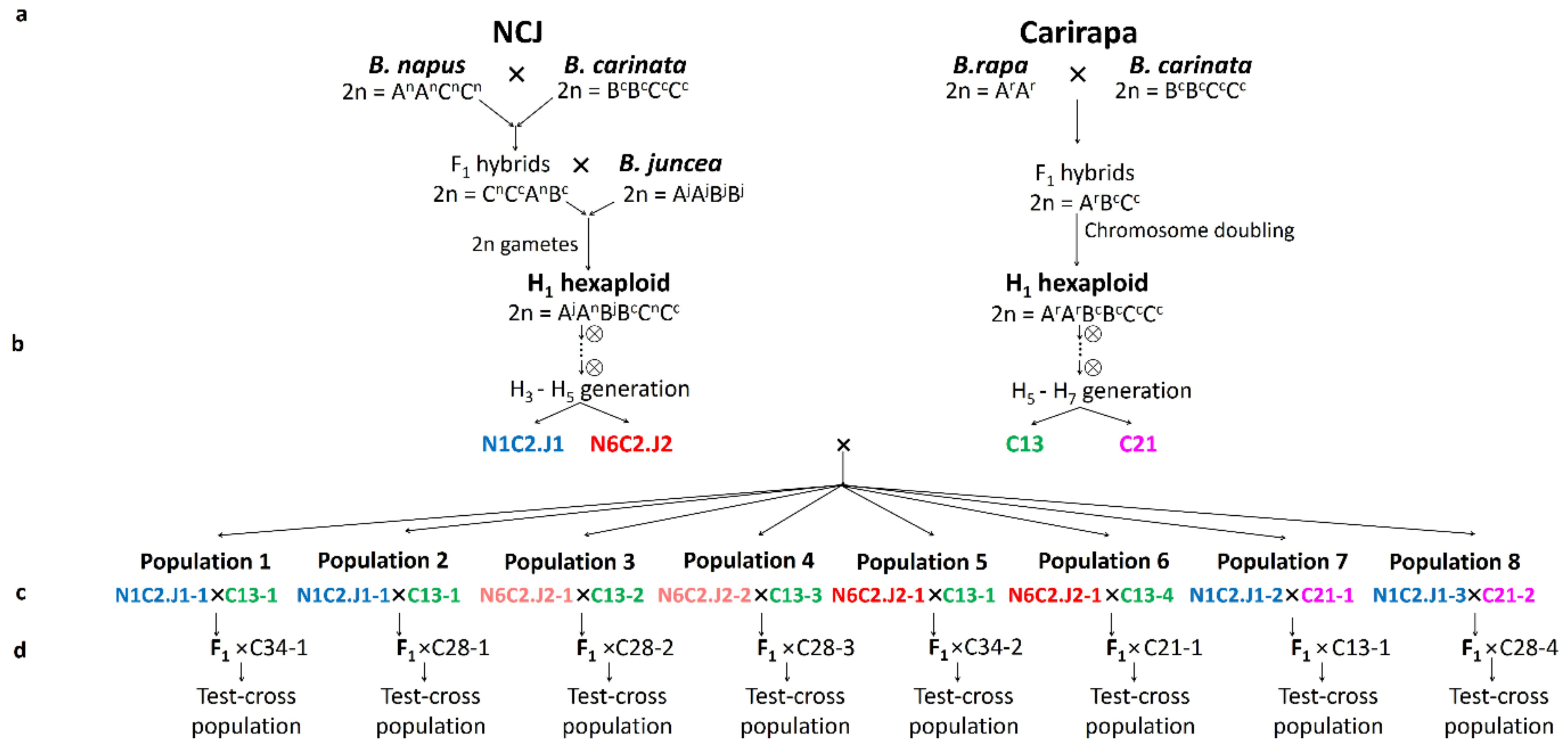


Figure 1. Crossing scheme. The arrows indicate the direction of the crossing. a Crossings involved in the production of the NCJ and carirapa hexaploid lines. b Selfing and selection of the genotypes. C Crossings between NCJ and carirapa genotypes. For the NCJ genotype combination N6C2.J2, two different lineages were used for crossing, indicated by a different shade of red. The number after the genotype combination represents the plant number. d F1 test-cross to another carirapa hexaploid.

3.3 Materials and methods

3.3.1 Plant material and crossing scheme

The most advanced genotypes available from the two NCJ parental genotype combinations N1C2.J1 and N6C2.J2 (one and two lineages, respectively) (Mason *et al.*, 2012; Mwathi *et al.*, 2017) and as well as one lineage from each carirapa allohexaploid genotypes C13, C21 (Tian *et al.*, 2010), were selected based on chromosome number and total seeds produced (Fig. 1b, Supplementary Table 1). In total, 12 plants were grown from the selected genotypes, with 6 plants per allohexaploid type: three plants N1C2.J1, two plants N6C2.J2 lineage one, one plant N6C2.J2 lineage two, four plants C13, and two plants C21 (Fig.1c). Initially, two plants from each of hexaploid genotypes C13 (plants 2 and 3) and N6C2.J2 (lineage one, plants 1 and 2) were grown and crossed under greenhouse conditions at Justus Liebig University, Giessen, Germany, while the rest of the plants were grown under field conditions at Huazhong Agricultural University, Wuhan, China. In the first round of crossings (F_1 hybrid production), the NCJ hexaploid plants were used as a female parent, and the carirapa plants were used as the pollen donor to produce eight different populations (Fig. 1c). The cross was done by hand, via emasculating flower buds and gently rubbing the anthers over the exposed stigma. The pollinated buds were then labeled and covered with a microperforated bag to prevent contamination from other pollen sources.

The F_1 hybrid seeds were collected and grown under field conditions at Huazhong Agricultural University, Wuhan, China. To be able to analyze the meiotic performance and allele segregation from this new F_1 hybrid, a test-cross was carried out. Selection of the F_1 hybrids was done based on qualitative phenotyping (e.g. high pollen production, relatively normal agronomic phenotype, plants looked like true hybrids) for individual plants resulting from the crosses between the lines. A total of eight F_1 hybrid plants were selected and test-crossed to another carirapa hexaploid (genotypes C21, C28 and C34) (Tian *et al.*, 2010) (Fig. 1d). The test cross-progeny varied in size depending on the population, ranging from 14-22 individuals that were grown under field conditions at Huazhong Agricultural University, Wuhan, China.

3.3.2 DNA extraction and SNP analysis

From each plant, a piece of leaf sample was collected and DNA was extracted using the CTAB method (Doyle & Doyle, 1990). For the following plants the original leaf material was not available but three different sibling plants were used instead: F_1 hybrid population 3, and test-cross parent population 4. In the case of *B. carinata* and *B. rapa* parental lines of the carirapa hexaploids, we extracted DNA from two different plants to account for potential heterosis in the material. The DNA was then genotyped using the Illumina Infinium *Brassica* 90K SNP array (Ilumina, San Diego CA, USA) following manufacturer´s instructions. A total of 77 970 SNPs distributed across the A (23 482), B (25 822), C (26 731), and unplaced location (1 877) *Brassica* subgenomes were obtained after applying the recommended cluster file for the A and C genomes (Clarke et al. 2016, Supplementary Data set Table 2) and by automated clustering in Genome Studio for the B genome. SNP positions for the A and C genomes were determined by the top hit (highest *e*-value) based on BLAST to the *B. napus* Darmor-*bzh* v. 8.1 reference genome (Bayer *et al.*, 2017). For the B genome, we used the positions provided in the Illumina Infinium 90K SNP array, which were based on the *B. nigra* Ni100 short read reference genome assembly (Perumal *et al.*, 2020). The SNP data was initially cleaned by removing non-specific alleles and SNPs with undetermined genomic locations.

The first step in the analysis was to carry out paternity testing to verify the genetic composition of the F_1 hybrid between the NCJ and carirapa allohexaploid parents for each of the eight populations. For this, homozygous polymorphic alleles for each parent in the B subgenome were used as diagnostics. If the expected heterozygosity was observed in the F_1 plant (e.g. NCJ allele AA × carirapa allele BB → F_1 hybrid allele AB) it was considered a true F_1 hybrid. The second step was to determine true segregating progeny between the F_1 hybrid and the test-cross carirapa allohexaploid parent. To do this, the same approach was used by selecting homozygous polymorphic alleles in the B subgenome between the F_1 and test-cross carirapa parent: if the segregating progeny had the expected heterozygous allele, they were considered true test-cross progeny. If the population were true hybrids in both analyzed cases, subsequent analyses were done.

3.3.3 Chromosome count and molecular karyotyping

To determine chromosome presence or absence, we used SNP and Log_2 R ratio data (Supplementary Data set Table 3). The absence of both chromosomes was seen across the entire chromosome, or most of it, as "no call" SNPs (NC). If the chromosome was present in the SNP data (at least one copy of the chromosome present), the Log_2 R ratio data was used to determine loss or gain of a chromosome copy by assessing the inheritance of the centromeric region of each chromosome (inheritance of the centromere was inferred to mean inheritance of that chromosome, since chromosome fragments cannot be transmitted without a centromere). Experimentally-derived values were used based on comparison to hybrid standards with known haploid and diploid chromosome complements. Log_2 R ratios between -0.5 and -0.2 were assumed to indicate one missing chromosome copy, Log_2 R ratios between 0.2 to 0.5 were assumed to indicate gain of an extra copy and Log_2 R ratios of more than 0.5 to indicate more than one extra chromosome copy gained. The approximate centromere locations for the A and C chromosomes were established according to the *B. napus* Darmor-*bzh* v8.1 reference genome (Bayer *et al.*, 2017), based on remapping of previous genetic data (Mason et al. 2016). For molecular karyotyping, a similar approach was used. If no-call (NC) SNPs covering ≥ 1 mega bases (Mb) in the chromosome was observed, it was categorized as both copies missing in that particular region of the chromosome. A similar size cut-off was used for the Log_2 R ratio values. Missing regions covering less than 1 Mb were not considered in the analysis due to SNP distribution and density constraints. If the duplication or deletion event was found in two copies (on both homologous chromosome sets), we classified it as "fixed" event, since this must have resulted from a previous meiosis, after which a self-pollination event allowed two gametes with the same rearrangement event to come together in the same plant. On the other hand, if a rearrangement was only present in one of the homologous chromosomes it was considered a segregating rearrangement. If a rearrangement was not present in either of the parents but it was detected in the next generation, it was classified as a *de novo* event. Putative translocations between the genomes were established based on initially scoring deletions/duplications based on the Log_2 R ration values in combination with the already known primary homoeology relationships between the A, B and C genomes (Chalhoub *et al.*, 2014; Perumal *et al.*, 2020) (Figure 2, Supplementary Table 4). The final karyotype was plotted in RStudio using the R package chromDraw (Janečka & Lysak, 2016) and posterior editing was done in GIMP ver. 2.10.20.

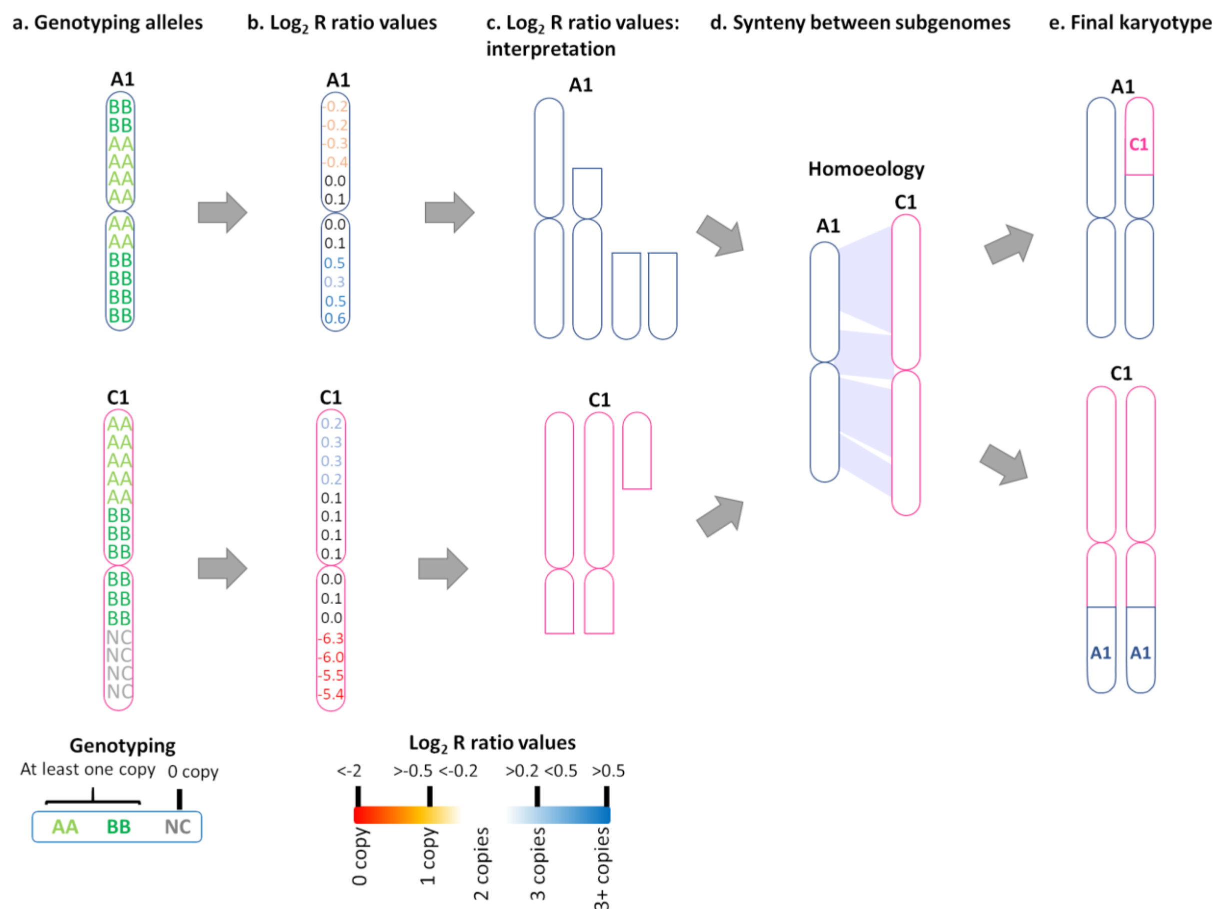


Figure 2. Example of the molecular karyotyping workflow. a. Genotype for chromosomes A1 and C1 in *Brassica* hexaploids: AA and BB: homozygous for allele A or B, respectively. NC, no-call. The end of the C1 chromosome is missing. b. Log2 R ratio values: values obtained from the SNP chip. Experimentally derived values were used to establish the ranges for each type of copy number variation (details in the legend). c. Log R ratio values interpretation. The top of chromosome A1 is present in a single copy, and the end of A1 has duplication. In the case of C1, there is duplication at the beginning of the chromosome and a deletion (both copies missing) at the end of the chromosome. The extra copies do not have a centromere; as chromosome fragments are eliminated in mitosis without a centromere, these fragments must be located in a chromosome. d Synteny between subgenomes: *Brassica* homology (Chalhoub *et al.*, 2014; Perumal *et al.*, 2020). Homologous chromosomes can pair during meiosis, and translocations can occur. In this case, the fragment sizes and positions of the different copy number variation events suggest translocation events between A1 and C1 chromosomes. e Final drawing of the karyotype based on genotyping, Log2 R ratio, and homology between the different subgenomes

3.3.4 Allele segregation in the F1 hybrid

To analyze the allele segregation from the F_1 hybrid, the alleles from each parent were analyzed independently. To analyze the NCJ parent, homozygous polymorphic alleles from the NCJ vs. carirapa parent 1/carirapa test-cross parent were filtered (NCJ allele: AA vs. carirapa parent 1/carirapa test-cross parent: BB), and vice versa for the carirapa parent. In each of the eight populations, the number of test-cross progeny ranged from 14 – 22 (Supplementary Table 1). The 1 : 1 AB : BB observed vs. expected allele segregation was tested using a X^2 test with a significance level of $p < 0.05$.

3.4 Results

3.4.1 Pre-existing fixed rearrangements in *Brassica* parental genotypes

Brassica genotypes used to produce the carirapa and NCJ allohexaploids were analyzed for fixed rearrangements in the genome. The parental genotypes used to produce the carirapa hexaploids, *B. carinata* accession "03949", and the *B. rapa* genotypes "Ankangzhong" and "WulitianYC" did not have any detectable fixed rearrangement events in their corresponding genomes. In the genotype "03949" we did observe a segregating event at the top of B01, where a single copy was missing between 0 and 13.6 Mb. In the case of the genotypes used in the crossing of NCJ hexaploids, we only observed fixed events in the *B. napus* genotypes. In the case of the *B. napus* genotype "Surpass400_024DH" (N1) we detected deletions in chromosomes A01 and A04, located at 2.2-4.7 Mb and 20.7-23-3 Mb, respectively. In the C genome of "Surpass400_024DH", we identified a deletion at the top of chromosome C01, located between 1.4 and 3.3 Mb. We also found two other deletion events located in chromosome C09, at positions 0-2 and 52.9-60.1 Mb, respectively. For the "Ag-Spectrum" genotype (N6) we observed a deletion on chromosome C02, located between 8-10.7 Mb.

3.4.2 Chromosome inheritance and fixed karyotype changes in NCJ and carirapa allohexaploid types

From the available NCJ and carirapa collection, the most advanced and fertile genotype combinations were selected. From the NCJ type, the genotypes selected were N1C2.J1 and N6C2.J2 and from the carirapa type, C13 and C21. The carirapa plants were not fully homozygous, suggesting that pollen cross-contamination with other genotypes may have occurred during propagation in the field. In the NCJ-type hybrids, residual heterozygosity was present based on the method of producing these hybrids, but no cross-contamination was indicated by the presence of alleles other than those from the parent genotypes. In total, six plants per allohexaploid type were grown and later on crossed to produce F_1 hybrids between the lines (Fig. 1c).

As the NCJ and carirapa allohexaploids have been independently selected by fertility (total seed number) through several generations, we analyzed the presence of fixed rearrangements (rearrangement present in both homologous chromosomes) in all the three genomes. To be able to have a better overview and frequency of all the fixed events, we combined them all into one figure (Fig. 3). A single fixed event involving the B genome was detected putatively between B01/A04, where the B01 segment was lost (deletion) and replaced by the end of A04 segment (duplication) for this homoeologous region. The end of A04 (~ 3Mb) was frequently lost, as was the case in seven out of 12 allohexaploid parents (four carirapa and three NCJ plants). However, this deletion event was already present in the *B. napus* cv. "Surpass400_024DH" used in the cross, and was inherited and fixed in the three NCJ plants analyzed. In the case of the carirapa plant C21-1 that had this deletion at the end of A04, the region was most likely replaced by the end of chromosome C04, but evidence of this was inconclusive in the NCJ plants analyzed.

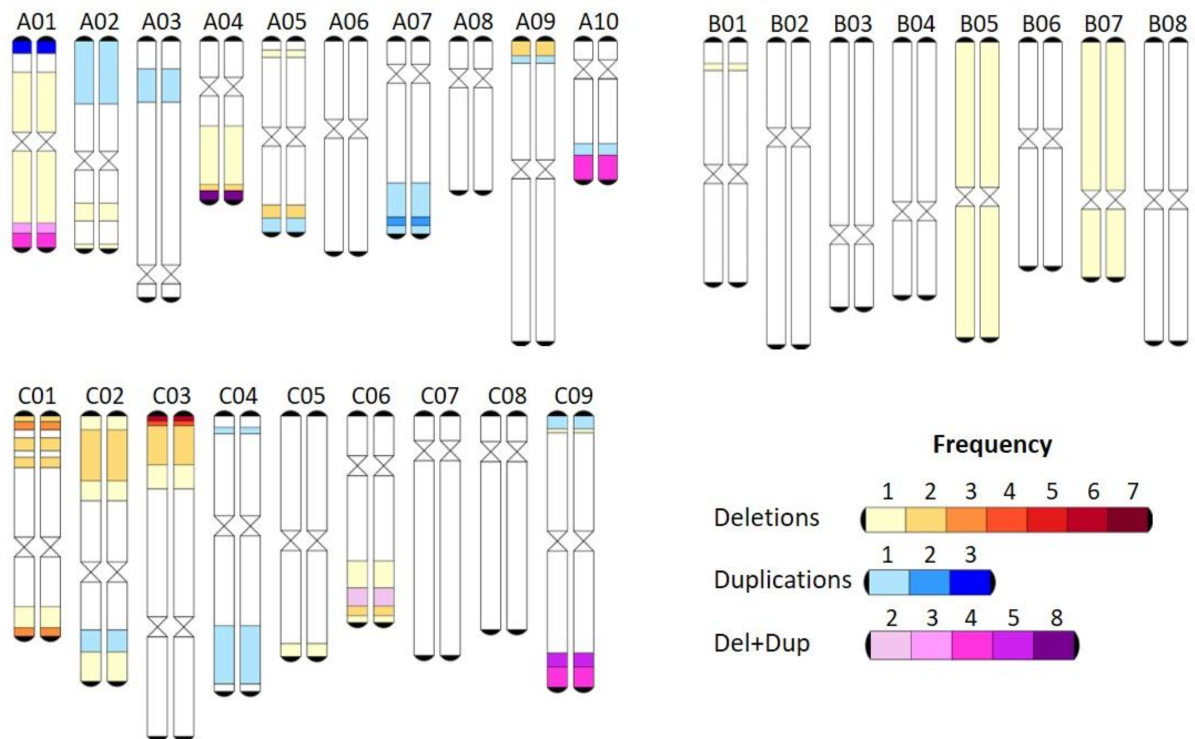


Figure 3. Karyotype of fixed events in *Brassica* allohexaploids NCJ (*B. napus* × *B. carinata* × *B. juncea*) and carirapa (*B. carinata* × *B. rapa*) type. The fixed partial or whole chromosome events are colored according to the legend for each of the three *Brassica* genomes.

For chromosomes B05 and B07, both copies were missing in the plants belonging to the two independent N6C2.J2 lineages. Another region that was frequently lost was located at the top of chromosome C03, ranging from approximately 20 – 1.2 Mb in size: this region was missing in all four carirapa C13 parents (representing four plants of one lineage). The three plants from the genotype combination N1C2.J1 (one lineage) had in common a duplication/deletion event involving an extra copy of the top of chromosome A01 (~ 2 Mb) being putatively translocated into chromosome C01. The deletion of C01 was already present in the *Brassica napus* cv. "Surpass400_024DH" (N1) used to produce the hexaploid, unlike the duplication of A01 which was a new rearrangement event. In some regions, we found an overlap of events (duplications and deletions) between the genotypes. The region at the end of chromosome A01 was duplicated in two carirapa C13 parents (one lineage), deleted in one carirapa parent (C21), and deleted in one NCJ parent (N6C2.J2). In the homoeologous region, there was no detectable duplication for any of the plants, hence it was more likely to be only a deletion event. Another region of overlap was found for putative homoeologous translocations between chromosomes

A10 and C09. The end of A10 had three duplication events (N1C2.J1) and one deletion event (C13), while C09 had one duplication (C13) and four deletions located at the end of C09 at ~ 53-60 Mb in three N1C2.J1 and, at ~ 52-55 Mb for one C13 parent. For the NCJ plants from the genotype combination N1C2.J1, the putative translocation between C09/A10 was already present in the *B. napus* cv. "Surpass400_024DH", and was inherited and fixed in the hexaploids. The rest of the events observed in the allohexaploids NCJ and carirapa were more parent-independent (occurred in only one plant). In total, we observed 50 fixed deletion events (32 carirapa, 18 NCJ) and 33 fixed duplication events (23 carirapa and 10 NCJ) involving the A, B and C genome of *Brassica* hexaploids. The fixed events observed in the carirapa hexaploids were not identified in either the *B. carinata* or the *B. rapa* accessions used as parents.

3.4.3 Crossing NCJ and carirapa allohexaploids: F1 hybrid and allele segregation

Seven out of eight F_1 hybrids were the result of a cross between carirapa and NCJ hexaploids, as expected (Table 1); one hybrid appeared to result from an outcross to an unknown paternal plant. The second step was to analyze the cross between the F_1 hybrid and the test-cross parent. To do this, the same approach as in the F_1 hybrid was used. In this case, populations 1 and 3 had only 7 and 5 individuals that corresponded to true test-cross progeny and the remaining individuals resulted from unintended self-pollination to produce F_2 progeny seeds. The other five populations were used to analyze allele segregation from the F_1 hybrid.

Table 1 *Brassica* allohexaploid populations. Genotypes crossed between carirapa (C13 and C21, H5-H7) and NCJ (N1C2.J1 and N6C2.J2, H3-H5) allohexaploid types. Plant number is also specified after the genotype code. Test-cross progeny correspond to the total number of progeny that were determined to comprise true test-cross progeny between the F_1 and the test-cross parent. *Populations removed from further analyses

Population	NCJ (female)	Carirapa (male)	Test-cross parent	Number of test-cross progeny
1*	N1C2.J1-1	C13-1	C34-1	7
2	N1C2.J1-1	C13-1	C28-1	18
3*	N6C2.J2-1	C13-2	C28-2	5
4	N6C2.J2-2	C13-3	C28-3	15
5	N6C2.J2-1	C13-1	C34-2	16
6	N6C2.J2-1	C13-4	C21-1	15
7*	N1C2.J1-2	C21-1	C13-1	Not true F_1
8	N1C2.J1-3	C21-2	C28-4	18

3.4.4 Allele segregating per population:

3.4.4.1 Population 2

The F_1 hybrid was the result of a combination between the genotypes N1C2.J1 × C13. This hybrid had 52 chromosomes distributed between the A genome (19), B genome (16) and C genome (17) (Fig. 4a). In the A genome, the chromosomes A01 and A05 were present in only a single copy, with just the chromosome from the carirapa parent present. Chromosome A01 was already a single chromosome in the NCJ parent, unlike A05, where both copies were present in the parent. The other chromosomes (A02-A09) were present in two copies, while chromosome A10 had at least one extra copy from the carirapa parent. In the A genome, putative non-reciprocal translocations between A05/C04, A05/C05, A09/C09 and A10/C09 were present in the chromosomes inherited from the carirapa parent. No translocations were observed in A-genome chromosomes from the NCJ parent. The bottom of chromosome A04 from NCJ was missing 2.5 Mb, corresponding to a known deletion present in the *B. napus* cv. "Surpass400_024DH" used as a parent in the crossing. The top of chromosome A09 from the NCJ parent was missing ~ 3Mb that corresponded partially to an extended deletion that was initially inherited from a deletion also present in the *B. napus* cv. "Surpass400_024DH". For the B genome, the F_1 hybrid contained the expected 16 chromosomes with no translocations or deleted regions detected. In the C genome, the chromosomes C01-C08 were correctly inherited

from the carirapa parent. In the case of chromosome C09, just a portion (~5 Mb) from the end of the chromosome was present, as a translocated region into chromosome A10. Part of the top of chromosome C03 (~7 Mb) was lost from the carirapa parent. The chromosomes C01-C09 were correctly inherited from the NCJ parent, despite the presence of only one copy of chromosome C02 in the parent. Putative translocations were observed in chromosomes coming from both parents. In the case of the carirapa parent chromosomes, the translocations present in the F_1 were C01/A01 and C02/A02, and in the NCJ parent chromosomes there were C01/A01, C05/A05, C06/A07, and C09/A10. From the above translocations, only two corresponded to putative *de novo* events identified in the F_1 hybrid.

Allele inheritance from the F_1 hybrid into the test-cross progeny was also analyzed (Fig. 4b). Segregation distortion was established if the observed allele segregation ratio was significantly different from the expected (X^2 test, $p < 0.05$). This test was performed independently for each of the parents of the F_1 hybrid. In population 2, 18 test-cross progeny individuals were used to assess allele segregation (for more details see materials and methods). The allele segregation from the A01 and C02 chromosomes could not be established due to the lack of enough polymorphic alleles. The segregation distortion observed in the chromosomes A05 and C09 corresponded directly to the presence of only a single chromosome in the carirapa or NCJ parent, respectively. The allele segregation distortion observed in A07, A09, A10, and C05 corresponded to rearrangements present in the F_1 hybrid. Allele segregation distortion not directly explained by the karyotype of the F_1 parent was observed in chromosomes A02 (extension of a known duplication present in the F_1 hybrid, carirapa), A03 (although few markers were represented, carirapa), C01 (NCJ), C04 (NCJ) and B02 (NCJ). Potentially, these events correspond to *de novo* rearrangements produced during meiosis in the F_1.

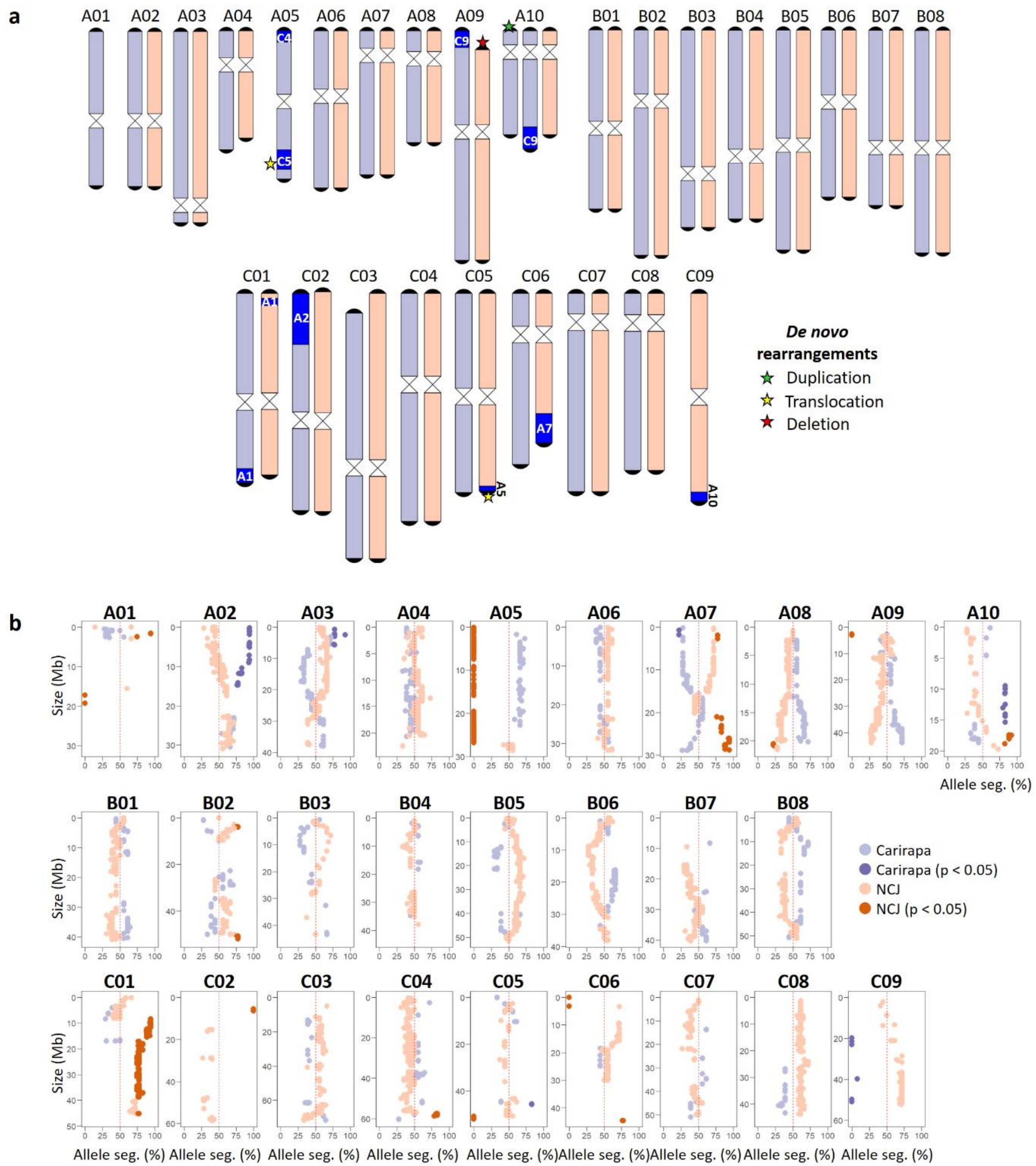


Figure 4. a Molecular karyotype and b allele segregation for F_1 hybrid population 2. Chromosomes are colored based on hexaploid parent: carirapa in purple, NCJ in orange. Rearrangements are colored in blue. *De novo* translocations (present in the F_1 hybrid but not in the parents) are marked with a star in a different color depending on the type (see legend). Chromosome sizes are represented in megabases (Mb). Expected segregation ratio of the alleles (50%) is marked with a red dotted line for each chromosome. Significant allele distortion (X^2 test, $p < 0.05$) is indicated with dark orange (NCJ) or dark purple (carirapa). Seg., segregation.

3.4.4.2 Population 4

The F_1 hybrid resulting from the combination between NCJ N6C2.J2 × carirapa C13 had 51 chromosomes distributed between the A (21), B (15) and C (15) genomes (Supplementary Fig. 2a). In the F_1 hybrid, chromosomes A02 and A07 had at least one extra copy from the carirapa and from the NCJ parent, respectively. These chromosomes were also doubled in the corresponding allohexaploid parent. Chromosome A04 was present as a single copy in the F_1 hybrid, inherited only from the NCJ parent. In the carirapa parent, chromosome A04 was present as a single copy and it was not inherited to the F_1. In the F_1, the chromosome A05 inherited from the carirapa parent had a small region missing (1.6 Mb) located at chromosome position ~24 Mb, and no potential candidate translocation corresponding to this region was identified. In the B genome, the F_1 hybrid had only one B07 chromosome (carirapa origin). Chromosome B07 was completely absent in the NCJ parent. In the B03 chromosome of the F_1 hybrid (NCJ origin) a duplicated region was identified, but it was not possible to determine the position in the genome of this extra copy (colored gray, see Supplementary Fig. 2a).

In the C genome, chromosomes C01, C02 and C06 were present in single copies (NCJ). The chromosomes C01 and C02 were also present as a single copy in the carirapa parent, unlike C06, which was present as two copies in the carirapa parent and as a single copy in the NCJ parent. In the A genome, putative translocations between homoeologs A01/C01, A02/C02, A07/C07 and A09/C08 were observed. In the B genome just one putative translocation between B01/A04 (NCJ) was observed. In the C genome, we had putative translocations involving C02/A02, C04/A04, C05/B01 and C07/B02.

In the analysis of the allele segregation, most significant distortions were explained by the translocations described above (Supplementary Fig. 2b). The exceptions were present in chromosomes A02 (carirapa), A07 (carirapa), A10 (carirapa), C06 (NCJ), B01 (carirapa) and B02 (NCJ). In addition, the single B07 chromosome in the F_1 hybrid was expected to be present in 50% of the test-cross population, but was only present in 18% of the individuals (3 out of 17).

3.4.4.3 Population 5

The F_1 hybrid (N6C2.J2 × C13) had 54 chromosomes distributed between the A (21), B (15) and C (18) genomes (Supplementary Fig. 3a). Chromosomes with at least one extra copy were present for A04 (NCJ) and C03 (NCJ). Chromosome A04 was already doubled in the NCJ parent, unlike chromosome C03, which resulted from a new chromosome duplication event. Single copies were observed for chromosomes B05 (carirapa) and C09 (NCJ). In the case of B05, both copies were missing in the NCJ parent. Chromosome C09 was present as a single copy in the carirapa parent, and did not get inherited into the F_1 hybrid. In the chromosomes from NCJ, putative translocations between A01/C01, A03/C03, A04/C04, B01/A05, C03/A03, and C08/A09 were found. In the case of chromosomes coming from the carirapa parent, we observed translocations involving the chromosomes A02/C02, A05/C04, A09/C09, C01/A01, C02/A10 and C03/A03 (Supplementary Fig. 3a).

In the population segregating for alleles from the F_1 hybrid, most of the allelic distortion was explained by CNV events in the parent F_1 (Supplementary Fig. 3b). The exceptions to this (putatively novel CNV events) were located in A02 (carirapa), A08 (NCJ and carirapa), B03 (NCJ), B04 (NCJ), B07 (NCJ), C02 (NCJ), C04 (NCJ and carirapa) and C08 (NCJ). Chromosome C09, as mentioned before, had just one copy in the F_1 hybrid and it was present in fewer test-cross individuals than expected (25% presence vs. 50% expected). In the case of B05 as a single chromosome, only a few polymorphic alleles were present with which to make a proper comparison, but it also seemed to be present less often than expected (25% presence vs. 50% expected).

3.4.4.4 Population 6

The F_1 hybrid in this population was a cross between N6C2.J2 × C13 and had 51 chromosomes distributed between the A (19), B (15) and C (17) genomes (Supplementary Fig. 4a). Single copy chromosomes were A04, B05 and C08. Chromosome B05 was already missing both copies in the NCJ parent. In the case of A04 and C08, both copies were present in the corresponding carirapa and NCJ parents, respectively. No extra chromosomes were observed. Putative translocations between A01/C01 and A09/C08 were detected in NCJ chromosomes. In the case

of the carirapa chromosomes, observed possible translocations were located between A09/C09-C08, C01/A01 and C02/A02.

When analyzing the allele segregation for alleles from the F_1 hybrid (Supplementary Fig. 4b), most of the distortion was explained by rearrangement events, with the exception of the following: end of A02 (carirapa), A03 (carirapa and NCJ), end of chromosome A04 (NCJ), A09 (carirapa), C01 (NCJ), C04 (NCJ), and C09 (NCJ). The A09 chromosome was a special case, as it was present in two copies in the F_1 hybrid, but in the test-cross population the A09 of carirapa origin was present at a higher frequency than expected (expected 50%, observed 86.6%). Interestingly, this chromosome had two translocations involving chromosomes C9 and C8 c. The single chromosomes A04 and C08 segregated as expected in the population, unlike B05, which was present in only two out of the 15 test-cross population individuals and not in half of them, as expected. No other changes were observed in the B genome.

3.4.4.5 Population 8

The cross between N1C2.J1 (euploid, 2n = 54) × C21 gave rise to an F_1 hybrid with 52 chromosomes distributed between the A (19), B (16) and C (17) genomes (Supplementary Fig. 5a). Single chromosomes were observed for A03 and C01. Both chromosomes were present as two copies in the corresponding NCJ and carirapa parents. No chromosomes were doubled. In the NCJ chromosomes, we observed potential translocations between A02/C02, C01/A01, C06/A07, and C09/A10. Chromosomes A04 and A09 inherited from the NCJ parent had a deletion at the bottom and at the top of the chromosome respectively. These deletions were already present in the parents and we did not observe a duplicated homoeologous region that could have replaced this fragment in the F_1 hybrid. In the chromosomes from the carirapa parent, we observed putative translocations between A01/C01, C02/A02, C03/A03 and C09/A09.

In the allele segregation from the F_1 hybrid (Supplementary Fig. 5b), most of the events were explained by the rearrangements, with the exception of areas on chromosomes A03 (NCJ), A06 (carirapa), B02 (NCJ), B06 (NCJ), B08 (NCJ and carirapa), C08 (NCJ) and C09 (NCJ).

3.5 De novo rearrangements and inheritance in the F1 hybrids

Overall, 50 new rearrangement events (22 duplications and 28 deletions) were observed in the F_1 hybrids. Out of these events, 28% (6 duplications and 8 deletions) were triggered by a previous event nearby or overlapping the chromosomic location of the new event already present in the hexaploid parent. On average, there were 10 new events per population, affecting mostly the A (52%) and C genome (44%). Both parents contributed almost equally to the *de novo* rearrangements observed in the F_1 hybrids, with the exception of population 5, where the NCJ parent contributed to 10 rearrangements compared to 4 coming from the carirapa parent. As mentioned before, the least affected genome was the B, where just two events in two populations were observed. In the A genome, the chromosome most affected by rearrangements was A09, with a total of 8 events (2 duplications and 6 deletions). In the case of the C genome, the chromosome with more *de novo* rearrangements was C02, with six events (2 duplications and 4 deletions). We also observed nine *de novo* events involving whole chromosomes, where six chromosomes were lost and three were present in an extra copy in the F_1 hybrids.

In the F_1 hybrids, we also observed that some of the rearrangement events present in the parental hexaploid plants were either inherited in the same size or reduced in size due to cross-overs. In total, 72 rearrangements were inherited from the hexaploid parental plants with an average of 14.4 rearrangement events per F_1 hybrid. Out of the 72 rearrangements, 8.3 % had a reduction in size due to a cross-over. Most of the events inherited from the parents corresponded to deletions (66.7 %), with seven events involving the loss of a chromosome, affecting mostly the C genome (27 events).

When analyzing the putatively *de novo* events produced by the F_1 hybrids, we observed a total of 43 events (8.6 events on average per population). Out of these events in the F_1 hybrids, 25 events were potential duplications and 18 deletions. Interestingly, many of these events also affected the B genome (9 events total, 8 from NCJ and 1 from carirapa origin, respectively). Overall, more events were produced by one meiosis in the F_1 hybrid than by meioses coming from the grandparents, although the difference was only significant between the carirapa parent and the F1s (Fig. 5, one way-ANOVA, $p < 0.05$).

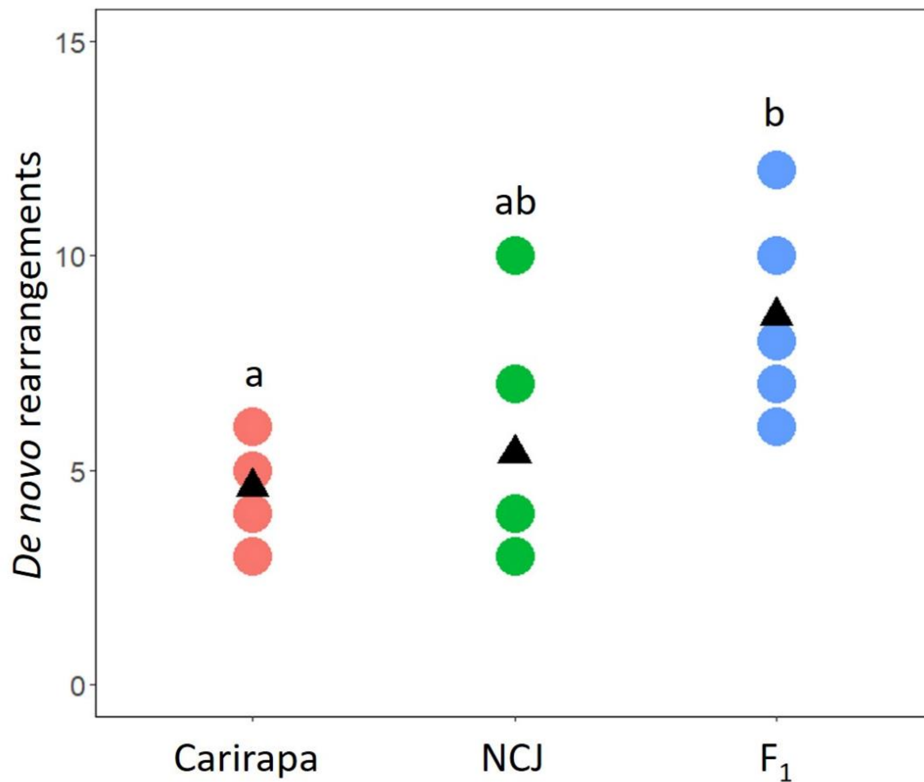


Figure 5. De novo rearrangements produced by meiosis in *Brassica* hexaploids carirapa (derived from *B. carinata* crossed with *B. rapa*), NCJ (derived from crosses between *B. napus*, *B.* carinata, and *B. juncea*) and their F_1 hybrids. Per group, there are 5 different meiosis events representing individual gametes produced from the parents and the F_1 (red: carirapa, green: NCJ and the F_1 hybrid between them: blue). The number of de novo rearrangements (occurring during this specific meiosis and not inherited from a previous meiotic event in the lineage) for each of these five meiotic events for the two parents and their hybrid is indicated as a dot. The average per group is drawn with a black triangle. Significant differences are indicated with letters (Tukey's HSD, $p < 0.05$): groups represented with a different letter (a, b) are significantly different, while groups that have a letter in com mon are not significantly different (a, ab and b, ab).

3.6 Discussion

From our study, it is clear that the new F_1 hybrids produced by the cross between carirapa and NCJ hexaploid lineages were able to tolerate inheritance of different chromosome rearrangements, despite the putative impact of these on meiosis. Initially, we hypothesized that meiosis might be more stable in the F_1 compared to the parents, as heterozygosity has been linked to crossover frequency in some species (Valenzuela *et al.*, 2012; Rowan *et al.*, 2019), but we observed the opposite. We observed an increase of rearrangements in the F_1 hybrid, which was significant compared to the carirapa parent (Fig. 5). An overall comparison may suggest that the more heterozygous the material, the higher the number of rearrangements that were produced, although further studies are needed to confirm this and to eliminate the confounding effect of the different genotypes. We also found more potential new rearrangements in the B genome of the F_1 hybrids, mostly affecting alleles inherited from the NCJ parent.

From our analysis, it was also clear that *Brassica* NCJ and carirapa allohexaploids can also accumulate and tolerate multiple structural rearrangements over generations (Fig. 3). This occurred even under selective pressure for improved fertility (number of total seeds produced), which hypothetically would be expected to select against aneuploid chromosome complements. This is in contrast to observations of *Brassica napus* synthetic lines in later generations ($S_{1:11}$), where fertility is majorly reduced in aneuploid lines compared to euploid lines (Xiong *et al.*, 2011). Chromosome loss similarly affected fertility in a *Brassica* hexaploid mapping population from carirapa origin, where 30 out of 51 plants were infertile if missing one chromosome (Yang *et al.*, 2018). However, Mason et al. (2014) also found no major effects of chromosome rearrangement on fertility in an NCJ allohexaploid-derived F_2 population.

The expected chromosome number from the hybrids analyzed in the present study was AABBCC = 2n = 54, but this was rarely observed (Supplementary Table 1). In the case of the carirapa plants, two of them had 54 chromosomes but were not euploid. The only NCJ plant with a euploid 2n = 54 chromosome complement failed to transmit a copy of chromosome A03 to the resulting F_1 hybrid, indicating that meiosis was still unstable in this plant. Our results may suggest that meiosis in these hybrids is still highly unstable, but that recurrent selection for fertile, viable plants is also acting to eliminate many of these rearrangements, so that plants move from euploid to aneuploid and back again, accumulating minor chromosome

rearrangements along the way. Aneuploidy and non-homologous recombination in polyploids and particularly synthetic polyploids are not rare events, and may in some cases even be beneficial due to the potential for creating novel phenotypic variation (reviewed in (Schiessl *et al.*, 2019)).

Nonhomologous recombination events reflected known chromosome relationships between the A, B and C genomes, as expected. Most translocations were observed between homologous chromosome pairs, as was previously observed in both natural and synthetic *B. napus* (Chalhoub *et al.*, 2014; Samans *et al.*, 2018) and in interspecific *Brassica* hybrids of various types as well as earlier generation NCJ allohexaploid populations (Mason *et al.*, 2014b; Gaebelein *et al.*, 2019b). For instance, greater changes were observed for homologous chromosomes A01/C01 and A02/C02, in accordance with results found in other synthetic *B. napus* lines (Gaeta *et al.*, 2007; Xiong *et al.*, 2011) and natural *B. napus* (Higgins *et al.*, 2018). The B genome was the least affected by fixed genomic rearrangements (Fig. 3) and all events observed in this genome were present only in the NCJ allohexaploid types. A possible explanation of why the B genome was more affected in the NCJ lines compared to the carirapa lines is the different method used to generate these hybrid types. The NCJ lines were produced using a two-step crossing method, where initially *B. napus* was crossed to *B. carinata* to form hybrids with genome compositions of 2n = CCAB = 36 (Mason et al. 2012). In these hybrids, the C genomes are present as homologous chromosomes, but the A and B genomes are haploid, and occasionally pair non-homologously with each other or with the C genome (Mason et al. 2010). As well, the preferential loss of the B genome over the A and C genome has been observed in other allohexaploid hybrids (Zhou *et al.*, 2016b). In the F_1 hybrids we observed very few rearrangements involving the B genome. In the F_1 hybrid parent of population 4 (Supplementary Fig. 2a), there were two events involving the introgression of B fragments into the C genome, with a carirapa origin. These events were already present as segregating events in the carirapa parent. Based on homology between the *Brassica* genomes (Perumal *et al.*, 2020) we identified the most likely position for these introgressions in the C genome. These events were very rare in all of the hexaploids analyzed, but may offer potential for introgression of chromosome fragments from the B genome carrying useful agronomic traits into the A or C genome for *Brassica napus* (rapeseed) crop improvement.

In the different F_1 hybrids analyzed we observed *de novo* rearrangements involving different chromosomes depending on the population. In some cases, these *de novo* rearrangements occurred close to regions where rearrangements were observed in the parental plants. As an example, in population 8, we observed an increase in the size of the deletion at the top of chromosome C03. This deletion was already present in the carirapa parent, but had a size of ~6.6 Mb, compared to 16.3 Mb in the F_1 hybrid (Supplementary Fig. 5). This suggests an increase in size for some of the rearrangements, although it was not the rule, as other translocations were directly inherited from the parent without size change. However, already-present translocations may lead to additional irregular pairing during meiosis, as recombinant chromosomes enforce close proximity between homoeologous pairs in translocation heterozygotes, facilitating the production of additional crossover events (Udall *et al.*, 2005) or it might be that those regions in general are more prone to recombine than others (De Muyt *et al.*, 2009). Mwathi et al. (2019) also observed this effect of pre-existing translocations in doubled-haploid NCJ allohexaploid lines, but due to the extreme instability of this population *de novo* translocation events were found to be much more common (2.2 events per plant on average) than events triggered by pre-existing rearrangements (0.8 events per plant on average). In our case, we observed on average 10 *de novo* rearrangements in the F_1 hybrids, and out of these, 2.8 events on average were proximal to or co-located with a pre-existing event. The difference observed between the two experiments might be attributed to the fact that in our case, the analyzed plants underwent more rounds of meiosis (H3-H7) compared to just two rounds for the Mwathi lines (H_2) (Mwathi *et al.*, 2019).

Although no fixed pattern of selection was observed across all the allohexaploid genotypes, several events notably seemed to occur in multiple independent lineages or to be preferentially selected for. This was observed in 3 plants belonging to the N1C2.J1 lineage where the beginning of chromosome A01 was doubled and putatively translocated into C01 (Fig. 3). The region translocated between A01/C01 has a length of ~ 2 Mb, and contains the meiosis gene *Cell Division Cycle 20* (*CDC20*). The gene *CDC20* has been found to be crucial in *Arabidopsis thaliana* meiosis, although it might have a different function in *Brassica* (Niu *et al.*, 2015).

The end of chromosome A04 was deleted (~ 1.1 – 2.5 Mb missing) in seven plants belonging to three independent lineages (two carirapa and one NCJ, Fig. 3). In the case of the NCJ plants, the deletion of this region was already present in the parental *B. napus* cv.

"Surpass400_024DH" and in the progenitor line "Surpass" (Higgins *et al.*, 2018). No doubling in the corresponding homoeologous region in C04 was observed for any of these lineages. Although no known meiosis genes fall in this region, the meiosis gene *ASYNAPTIC4* (*ASY4*) is located 2 Mb upstream of the deleted region of A04. In *Arabidopsis thaliana*, this protein is necessary to complete synapsis, cross-over formation and normal localization of interacting proteins ASY1 and ASY3 (Chambon *et al.*, 2018). Interestingly, the homozygous presence of a *ASY3* allele with a tandem duplication region has been associated with more stable autotetraploid *Arabidopsis lyrata* lines, as it causes a reduction in multivalent formation (Seear *et al.*, 2020a). The homoeologous region in C04 has recently been associated with fertility in synthetic *B. napus,* where a deletion of the last 1.5 Mb of chromosome C04 reduced seed number (Ferreira de Carvalho *et al.*, 2021). However, this deletion was not associated with a reduction in the number of non-homologous recombination events, suggesting that this effect may only be related to selection for fertility and not to increased stabilization of the genome. The top of chromosome C03 (~1,2 - 2.3 Mb) was also missing in four plants from the same carirapa lineage (C13, Fig. 3), most likely as the result of an event fixed very early on in this lineage, although the four plants differed slightly in the size of the deletion. This A03-C03 region has previously been identified in hexaploid lines as a translocation QTL associated with total seed number in NCJ hexaploids, where the top of chromosome C03 was replaced by a copy of A03 (Gaebelein *et al.*, 2019b). Within this region, we find a potential meiotic candidate gene called *BRCA2.* In *Arabidopsis*, the protein BRCA2 interacts in vitro with DMC1 (Dray *et al.*, 2006) and plays a crucial role in homologous recombination (Seeliger *et al.*, 2012).

Five populations from different crosses were analyzed for bias in allele segregation and chromosome inheritance. The B genome was least affected overall, although it was preferentially lost if present as single chromosomes (B05 and B07 in populations 4, 5, and 6). A similar case was observed for the single C09 chromosome (population 5, Supplementary Fig. 3a), that was also present less often than expected in one test-cross population. However, this was not an effect seen for all chromosomes present in a single copy: the majority (A03, A04, A05, C01, C02, C06, C08 and C09 in the different populations) were inherited as expected in the progeny (50%). Interestingly, in population 6, there was segregation distortion towards retention of chromosome A09 from the carirapa allohexaploid parent, although both chromosomes were present in the hybrid (Supplementary Fig. 4b). This chromosome inherited from the carirapa parent had putative translocations between chromosomes A09/C09 and

A09/C08. Additionally, the chromosome A09 from carirapa had a translocated C09 region of 1.7 Mb, containing the meiosis gene *X-ray induced 1* (*XRI1*). This gene has been shown to be involved in meiosis and post-meiotic stages of male gametes development in plants (Dean *et al.*, 2009). In this F_1 hybrid, we also just had one C08 chromosome (NCJ), and potentially the retention of an extra A09 occurred as a trisomy compensation of the missing homoeologous region. Compensatory trisomy of chromosomes has been previously described in resynthesized *B. napus* (e.g. trisomy of C05 chromosome, single copy A05)(Xiong *et al.*, 2011). The gain of extra chromosomes to compensate the loss of a homoeologous chromosome can help to prevent further chromosomal instability by providing a partner for the single chromosome (e.g. monosomic-trisomic substitutions) and by preventing the deleterious effects of aneuploidy and changes in gene balance (Xiong *et al.*, 2011).

In the test-cross populations 5 and 6, the end of chromosome A08 had an allele segregation distortion towards the allele inherited from the carirapa parent (Supplementary Fig. 3b and 4b). This region was ~1.3 Mb in size and contained the meiosis genes *ADA2b* and *RAD51D.* Both populations shared the same carirapa and NCJ genotype combination (C13 and N6C2.J2). In rice, *RAD51D* has been shown to be associated with the prevention of non-homoeologous recombination during meiosis (Zhang et al., 2020). The preferential inheritance of an allele putatively related to increased meiotic stability from *B. rapa* over an allele from *B. napus* is unexpected, as *B. napus* is an established allopolyploid species with good meiotic control. However, at least one *B. rapa* genotype has previously been identified to contain allelic variation relevant for meiotic stability in allohexaploids with *B. carinata* (Gupta et al. 2016). Further confirmation is needed to support this observation.

In test-cross population 4, allele inheritance distortion was observed at the end of A07, where alleles from the carirapa parent were preferentially inherited over alleles from the NCJ allohexaploids (Supplementary Fig. 2b). The region has a size of ~5.6 Mb and has also been previously associated with total seed number in an NCJ population (Gaebelein *et al.*, 2019b), although it is hard to point to a meiosis candidate gene as several can be found within this interval.

3.7 Conclusions

Our results show that *Brassica* carirapa and NCJ allohexaploids are highly tolerant of chromosome duplication and deletion events. No reproducible patterns of karyotype change were observed, although certain rearrangement events were present in more than one genotype. At the same time, no strong evidence of subgenome dominance was observed, although fewer rearrangements were found in the B subgenomes, as expected due to the lower degree of homology between the B compared to the A/C subgenomes (Perumal *et al.*, 2020). Although genomic stability has not yet been achieved, the new allohexaploid material could be intercrossed between lineages without major effects on meiosis. This suggests the opportunity to use this material in rapeseed breeding programs, with special interest in the different translocations observed. At the same time, intercrossing between lineages offers great potential to increase genetic diversity in our material, and may in future lead to allelic combinations that might be selected for to provide meiotic stability and fertility in a new *Brassica* allohexaploid crop.

4. Karyotype instability varies by species and genotype combination in allohexaploid *Brassica*

4.1 Abstract

Synthetic *Brassica* allohexaploids (2n = AABBCC) do not exist naturally but can be produced between six different parent species combinations, and can be used to investigate processes of polyploid formation and genome stabilization. In this study, we investigated hybridization potential, accumulation and frequency of copy number variants (CNVs), fertility, and karyotype stability in advanced generations of diverse allohexaploid genotypes belonging to different *Brassica* allohexaploid species combinations (NCJ types: *B. napus* × *B. carinata* × *B. juncea*; junleracea types*: B. juncea* × *B. oleracea*; naponigra types*: B. napus* × *B. nigra*; and carirapa types*: B. rapa* × *B. carinata*). Only 3.2% of allohexaploid plants investigated were euploids, with high frequencies of rearrangements. However, significant differences between genotypes and between lineages within parent genotype combinations were found for frequencies of euploids and rearrangements, with one NCJ line showing relatively high karyotype stability. Rearrangements were usually translocation events resulting from homoeologous exchange between the A and C genomes: twelve different chromosome pairs were involved in A→C or C→A duplication/deletion events. Increasing numbers of CNVs were significantly associated with fewer seeds per plant in NCJ allohexaploids (r = -0.43) but no significant association between rearrangements and fertility was found for either carirapa or junleracea types. Hybridization between different allohexaploids was mostly achievable, with 0 - 4.6 seeds per flower bud on average, and with a strong maternal effect depending on the genotype used. Novel hybrids between allohexaploid lineages showed similar fertility and stability to their parents. In the novel hybrid population, a significant correlation was observed between the inheritance of A-genome chromosome fragments (relative to C-genome fragments) and the total number of seeds produced per plant (r = 0.24). Our results suggest that synthetic *Brassica* allohexaploids can develop genomic stability, but that this occurs at very low frequencies, and may not always be under selective pressure due to the unpredictable relationship between fertility and genome composition in these hybrid types.

4.2 Introduction

Polyploidy is widely distributed in flowering plants, with recurrent whole genome duplication events dating back millions of years (Jiao *et al.*, 2011). In general, there are two major polyploid types: autopolyploids (origin of additional chromosome sets from within the same species or even individual) and allopolyploids (two or more different chromosome sets from different species). Many crops are successful polyploids, such as wheat, oats, and rapeseed (Wendel, 2000). Polyploidy can also have advantages such as increased cell size, growth rate, or organ size (Otto & Whitton, 2000), fixed heterosis and gene redundancy (Comai, 2005), and different soil and climate adaptations (Mousavizadeh *et al.*, 2022). Unfortunately, many newly synthesized polyploids tend to be less fit, mostly due to genome instability in the context of meiosis (Pelé *et al.*, 2018). In sexually reproducing plants, meiosis plays a fundamental role in ensuring correct chromosome recombination and inheritance into the next generations. In polyploids, the meiosis process becomes more convoluted since there are more possible pairing options per chromosome. Hence, the most common meiotic abnormality during meiosis in polyploids corresponds to chromosome associations involving multiple homologs or homoeologs (Grandont *et al.*, 2013b). These kinds of meiotic irregularities can lead to copy number variants (CNVs), aneuploid progeny, and non-viable gametes, making the stabilization of new polyploids a major challenge.

Studies trying to understand how polyploids might stabilize meiosis have been done in many species, and major loci and genes have been identified in Arabidopsis (e.g. (Yant *et al.*, 2013; Seear *et al.*, 2020b)), wheat (Sears & Okamoto, 1958; Griffiths *et al.*, 2006; Bhullar *et al.*, 2014; Rey *et al.*, 2017), and *Brassica* (Jenczewski *et al.*, 2003; Gaebelein *et al.*, 2019b; Higgins *et al.*, 2021), among others. In wheat polyploids, a major locus controlling homoeologous pairing has been identified as *Ph1* (*Pairing homoeologous 1*) located on chromosome 5B. In *B. napus* haploids (2n = AC), a major locus, *PrBn,* was identified as responsible for variation in pairing behavior (Jenczewski *et al.*, 2003), although a similar effect of this locus was not observed in allotetraploid plants (Grandont *et al.*, 2014). In a segregating *B. napus* population where homoeologous recombination events were quantified, three quantitative trait loci (QTL) were found on chromosomes A03 (23.3 - 26.3 Mb), A09 (11.1 - 23.9 Mb), and C07 (42.2 - 43.4 Mb) (Higgins *et al.*, 2021). The QTL located on chromosome A09 accounted for up to 58% of the variability observed in homoeologous pairing (Higgins *et al.*, 2021). When comparing gene

expression using RNA-seq data collected from meiocytes, an ortholog of the meiotic gene *RPA1C* was found to be a candidate based on differential gene expression analysis (Higgins *et al.*, 2021). In allohexaploid *Brassica* produced from the cross [(*B. napus* × *B. carinata*) × *B. juncea*], several QTL were identified on chromosomes A03, A04, A10, B05, B06, B08, and C03 that were putatively associated with meiotic behaviour (Gaebelein *et al.*, 2019b).

Trigenomic allohexaploid *Brassica* (2n = 54 = AABBCC) do not exist in nature but can be produced via different cross combinations of the diploid and allotetraploid species (Gaebelein & Mason, 2018). In the *Brassica* genus the diploid species *B. oleracea* (2n = 18 = CC), *B. rapa* (2n = 20 = AA), and *B. nigra* (2n = 16 = BB) hybridized to form the allotetraploid species *B. napus* (2n = 38 = AACC), *B. carinata* (2n = 34 = BBCC), and *B. juncea* (2n = 36 = AABB) (U, 1935). The most common method to produce allohexaploids is the cross between *B. carinata* × *B. rapa* followed by induced chromosome doubling (e.g. via colchicine treatment), and this new synthetic type is known as "carirapa" (Tian *et al.*, 2010; Zou *et al.*, 2010; Gaebelein & Mason, 2018). It is also possible to cross between *B. juncea* × *B. oleracea,* also followed by induced chromosome doubling (e.g. (Mwathi *et al.*, 2019)), where the final hybrid is known as "junleracea", or between *B. napus* × *B. nigra* followed by induced chromosome doubling to produce "naponigra" hexaploids. A fairly new method to produce allohexaploids relies on unreduced gamete production in a two-step hybridization method between [(*B. napus* × *B. carinata*) × *B. juncea*], referred to as "NCJ" types (Mason *et al.*, 2012). Although trigenomic *Brassica* allohexaploids can be made in many species combinations, the number of genotype combinations which have successfully produced a hybrid is very limited. To produce a trigenomic *Brassica* allohexaploid two or more different species are usually crossed and the success might depend on factors such as the direction of the cross, the ploidy of the parents, species origin (Morgan *et al.*, 2021), temperature, and genetic variation (Bjerkan *et al.*, 2020) on top of the pre- and post-fertilization barriers. Up until now, crosses between different allohexaploid types have not been produced or investigated. If crosses between different allohexaploid types can be achieved, this could help to increase genetic diversity in the limited existing material.

Synthetic allohexaploid *Brassica* are usually meiotically unstable to a greater or lesser degree, with strong genotype-specific effects (Gaebelein *et al.*, 2019b). Meiotic instability resulting in non-homologous chromosome recombination and aneuploidy has been observed in the initial

generations of each allohexaploid type in the majority of genotypes tested so far (Tian *et al.*, 2010; Mason *et al.*, 2014b; Gupta *et al.*, 2016; Zhou *et al.*, 2016b; Gaebelein *et al.*, 2019a,b; Mwathi *et al.*, 2020). To date, very high meiotic stability (as assessed by bivalent frequency) has only been observed in a carirapa allohexaploid derived from one specific *B. rapa* cultivar (Gupta *et al.*, 2016). There is also evidence for relatively high stability (as assessed by generational frequency of 2n = 54 chromosome complements) in several other carirapa lines, although this was rare in a large set of characterized genotypes (Tian et al. 2010). There is also some evidence in different types of hexaploids for a generational increment in meiotic stability (Zhou *et al.*, 2016b). However, other studies suggest that aneuploid plants have a tendency to produce more aneuploid offspring (Iwasa, 1964), and that existing non-homologous recombination events may also cause problems in subsequent generations (Mason *et al.*, 2015; Mwathi *et al.*, 2019). Despite previous work on this topic, it has to date been difficult to systematically compare genomic stability between allohexaploid combinations and genotypes, due to the wide range of different methodologies used to assess this.

In this study, we aimed to compare genome stability between eight genotypes (nine lineages) of different synthetic *Brassica* hexaploids resulting from three cross combinations type by assessing the frequency of novel CNVs arising in a single generation between sibling plants, in order to comprehensively determine differences between lines. We also assessed if karyotype changes were reproducible or random across the allohexaploid genomes after polyploidisation. We further aimed to determine how feasible it is to combine new *Brassica* allohexaploid types via the success of hand-crossing between and within four allohexaploid types and the fertility and karyotype stability of resulting progeny by assessing CNVs in the progeny.

4.3 Materials and methods

4.3.1 Plant material:

Parental *Brassica* genotypes used to produce NCJ, junleracea, and naponigra allohexaploids were *B. napus* “Surpass400_024DH”, “Boomer”, “Ag-Spectrum”, “MSL Express”, and “Ningyou7”, from here on referred to as N1, N5, N6, N8, and N9 respectively; *B. carinata*

“195923.3.2_01DH”, and “94024.2_02DH” (C1 and C2 respectively); and *B. juncea* genotypes “JN9-04”, “Purple leaf mustard”, and “B578” (J1, J2, and J3, respectively). *Brassica oleracea* genotype “TO1000” is referred to as O1, and *B. nigra* genotypes “Junius”, “IX7”, and “IX13”are referred to as I1, I2, and I3, respectively (Mason *et al.*, 2010, 2012; Gaebelein *et al.*, 2019a; Mwathi *et al.*, 2020). Carirapa hexaploid parental genotypes used in the cross are as follows: *B. carinata* “CGN03943” × *B. rapa* “CGN03907” produced carirapa C05, *B. carinata* “CGN03949” × *B. rapa* “Ankangzhong” produced carirapa C13, and a cross between two different carirapa - C21 (*B. carinata* “CGN03983” × *B. rapa* “Wulitian YC”) and C28 (*B. carinata* “CGN03995” × *B. rapa* “Baijian 13”) - produced carirapa C2128 (Tian *et al.*, 2010) .

4.3.2 Genome stability analysis:

The following *Brassica* parental genotypes were selected as controls: N1, N5, C1, C2, J1, J2, and O1. *Brassica* hexaploid genotypes were selected based on total seed number. From the NCJ type, one lineage from the N1C1.J1 genotype (generations H_{5-6}), N1C2.J1 (generation H_6), N6C2.J2 (generations H_{5-6}) and two lineages from genotype N5C2.J1 (lineages described as a and b at the end of the genotype name, generations H_5 and H_4, respectively) were selected (Mason *et al.*, 2012). From the carirapa type, one lineage per genotypes C05 (generation H_{10}), C13 (generation H_{8-9}), C2128 (generation H_6) were chosen (Tian *et al.*, 2010). From the junleracea type, one lineage of genotype J3O1 (Mwathi *et al.*, 2020) was selected (H_4 generation). From each *Brassica* control, five plants were grown (35 plants in total). In the case of the allohexaploids NCJ and carirapa, five different sibling lines per lineage were selected four to five plants per line were grown, giving a total of 24-25 per lineage, with a total of 124 plants from NCJ and 74 plants from carirapa. In the case of the junleracea type, four different sibling lines were selected and five plants were grown per sibling line, with a total of 20 plants (Supplementary Table 1).

4.3.3 Crossings between four allohexaploid types:

The following NCJ combinations were selected: N1C1.J1 (seven plants, H_3 generation), N1C2.J1 (seven plants, H_4 generation), N5C2.J2a (nine plants, H_3 generation), N5C2.J2b (five plants, H_2 generation), N6C2.J2 (nine plants, H_3 generation), N4C2.J1 (eight plants, H_1 generation), N5C2.J1 (three plants, H_2 generation), and N7C1.J1 (five plants, H_2 generation) (Mason *et al.*, 2012). The *Brassica* carirapa allohexaploids selected were C05 (10 plants, H_8 generation), C13 (seven plants, H_6 generation), and C2128 (five plants, H_4 generation) (Tian *et al.*, 2010).

Cuttings of *Brassica* naponigra were used for crossing. The selected genotype combinations were N8.I1 (two cuttings), N8.I2 (two cuttings), N8.I3 (three cuttings), N5.I2 (four cuttings), and N9.I3 (four cuttings) (Gaebelein *et al.*, 2019a). One genotype from *Brassica* junleracea was selected for crossing: J3O1 (five plants, H_2 generation) (Mwathi *et al.*, 2020).

4.3.4 F_1 hybrids and parents:

Brassica F_1 hybrids produced from crosses between different allohexaploid types were selected based on the number of seeds produced and the genotype combination. Allohexaploid genotypes selected and the individual parent plants that were used to produce the F_1 hybrids were as follows: N1C1.J1 × N6C2.J2 (plant #2), N6C2.J2 (plant #3) × N5.I2, N6C2.J2 (plant #1) × C13 (plant #1), N6C2.J2 (plant #1)× J3O1 (plant #1), C13 (plant #2) × C05, C13 (plant #2) × J3O1 (plant #1), C13 (plant #3) × N8.I3, and N8.I3 × J3O1 (plant #2). Three plants were grown from each of the parent genotypes and F_1 hybrid combinations for further analysis.

4.3.5 Plant growth conditions, fertility, and plant data collection:

Seeds were germinated in quick pot trays under greenhouse conditions at Justus-Liebig University (Giessen), then transferred into 1 L pots after reaching the two to three true-leaf

stage. Plants used for the genome stability assay were grown only in quick pots until discarded. Plants were watered as required with weekly fertilization, and were grown under a photoperiod of 16 h day and 8 h night in a temperature-controlled chamber. Plant protection was applied to control pests and diseases as needed. Days to flowering was measured by counting from the day of sowing until the first flower opened (developmental stage BBCH60).

Pollen viability was estimated by using two flowers per plant using an Amphasys Z30 (Amphasys AG, Switzerland) with the F chip and AF7 buffer, following provider´s recommendations (Supplementary Table 2). Pollen viability was also established by collecting two flowers with mature pollen and placing the pollen grain of each flower (6 anthers) on a glass microscope slide, staining it with acetocarmine 1% (1 g of carmine powder in 100 mL of 45% acetic acid) followed by visualizing under the microscope (Supplementary Table 2). For acetocarmine staining, 300 pollen grains were counted per flower, where round and red-stained pollen grains were considered alive and shrunken, yellow or unstained pollen grains were counted as dead. In both pollen viability estimates, the value was expressed as a percentage viability based on the relative numbers of live and dead pollen grains per sample. The average pollen viability was established based on two flowers counted for each plant.

The total number of self-pollinated seeds produced per plant for the parents and F_1 hybrids was obtained by covering the entire plant with a microperforated bag (0.5 mm, Crispac-Beutel, Baumann Saatzuchtbedarf GmbH, Waldenburg, Germany) after the start of flowering, and keeping the bags on the plant until seed harvesting.

4.3.6 Crossing success:

Once at the flowering stage, the different genotypes were crossed in one direction (Supplementary Table 3). In total, we aimed to produced 126 different crosses with 100 flower buds per cross combination (12 000 flower buds in total) out of which 40 crosses were between NCJ and naponigra, 24 crosses between NCJ and carirapa, eight crosses between NCJ and junleracea, five crosses between junleracea and naponigra, 15 crosses between naponigra and carirapa, three crosses between junleracea and carirapa, 28 crosses between NCJ, and three crosses between carirapa genotypes. The female or male parent plant used in each of the

crosses was selected based on phenotypic observations of pollen availability, number and size of flower buds, and overall fitness of the plant. The female parent plant was selected first, based on relative number of inflorescences and relative number of flower buds compared to the other genotype involved in the cross, where the plant with the highest numbers of both was selected as the female parent. In crosses where one parent plant or all plants of a specific genotype produced very little to no pollen, these were also selected as the female parent. The male parent was selected as a default in each combination after the female parent was chosen. Some plants produced both high numbers of flower buds and large amounts of mature pollen, and were used as both the female and male parent in different crosses.

Crosses were done by using forceps to open the flower buds from the female parent to remove the anthers and to expose the stigma. Then, mature anthers from the selected male parent were collected and gently rubbed over the female parent stigma. The cross was then labeled and covered with a microperforated plastic bag (0.5 mm, Crispac-Beutel, Baumann Saatzuchtbedarf GmbH, Waldenburg, Germany) until harvest to prevent contamination with external pollen donors. The female parent is the first genotype named in the corresponding cross combination.

In parallel, 1-10 branches per plant were covered with microperforated bags to produce self-pollinated seeds. After seeds in the crossing and self-pollination bags were finished ripening, total siliques developed, number of seeds per silique, viviparous seeds (germinated seeds inside the silique), and total seeds (normal seeds plus viviparous seeds) were counted. The ratio of crossing success was calculated by dividing the total number of seeds obtained by the total number of flower buds crossed. The number of siliques that developed after pollination (the number of instances when after pollination the silique elongated and putatively developed seeds, unlike undeveloped siliques that died and fell off the plant a few days after pollination) were also quantified.

4.3.7 DNA extraction and SNP genotyping:

After the plants reached the three to four true-leaves stage, approximately 100 mg of leaf material was collected and stored at -20°C until processing. DNA was extracted using the CTAB

method (Doyle & Doyle, 1990). The samples were then treated with RNAse (Carl Roth, Germany) according to manufacturer's instructions. DNA was quantified using a Qubit fluorometer and dsDNA BR assay kit (Thermofisher Scientific, Germany) following manufacturer´s instructions. DNA quality was also checked using agarose gel electrophoresis.

Lyophilized DNA samples were sent for genotyping using the Illumina Infinium *Brassica* 90K SNP array (Illumina, USA) following the manufacturer´s instructions. The initial filtering of the data and analysis was done as previously described (Quezada-Martinez *et al.*, 2022). Briefly, in Genome Studio 2.0 (Illumina, USA) the analysis of the A and C genome SNPs was done using the recommended cluster file (Clarke *et al.*, 2016) and automated clustering was used for the B genome. The top-hit (highest *e*-value) was determined for the A and C genome probes based on a BLAST to the *B. napus* Darmor-*bzh* v. 8.1 reference genome (Bayer *et al.*, 2017). The positions used for the B genome probe were the ones provided in the Illumina Infinium 90K SNP array, based on an early version of the short-read *B. nigra* genome Ni100 (Perumal *et al.*, 2020). The initial cleaning of the data involved the removal of non-specific SNPs and those SNPs where the genomic location was not able to be determined. SNPs with 100% no-calls across all individuals were also removed from further analysis. A total of 42 554 SNP markers were kept for further analysis, distributed across the A (12 577 SNPs), C (17 867 SNPs), and B (12 111 SNPs) subgenomes (Supplementary Table 4).

4.3.8 Copy number variant analysis:

To determine the presence or absence of a chromosome we used SNP data combined with Log_2 R ratio values (see method as described in (Quezada-Martinez *et al.*, 2022)). The approximate centromere locations for the A and C chromosomes were based on the *B. napus* Darmor-*bzh* v. 8.1 reference genome (Bayer *et al.*, 2017) using estimates remapped from (Mason *et al.*, 2016). For the B genome, the approximate centromeric locations were taken from the *B. nigra* Ni100 genome (Perumal *et al.*, 2020).

Copy number variantion was established based on experimentally-derived values as described in (Quezada-Martinez *et al.*, 2022). Fixed events for duplications and deletions were classified

as such if the event was observed for both homologs. If the rearrangement event was heterozygous, showing as present in only one homolog, the event was classified as segregating.

Initially, all copy number events were scored independently by chromosome and by plant. Secondarily, potential translocation events were assessed based on known homoeologous relationships between the subgenomes (Chalhoub *et al.*, 2014; Perumal *et al.*, 2020). For example, once a duplication event was observed, the corresponding homoeologous region was also looked at: if the top of A01 had a 2 Mb duplication, the primary homeologous region at the top of C01 was checked, and if a deletion or missing copy was present in this region the event was classified as a translocation. The final karyotype was drawn using the R package chromDraw (Janečka & Lysak, 2016) in RStudio v.2022.07.1 and later modified using GIMP v.2.10.20.

4.3.9 Statistical analysis:

Multiple regression models were used to determine the effects of independent variables on the number of seeds produced per number of flower buds crossed (crossing success ratio). To account for heteroscedasticity in our model (right-skewed residuals, Breusch-Pagan test p = 0.005208), the final model was calculated using weights. Initially, a multiple regression model including all the variables was created (allohexaploid type combination, genotype of female parent, genotype of male parent, and pollen viability). After initial analysis of single term effects, pollen viability had no statistically significant effect (p = 0.06247) and it was removed from the final model. The new model incorporated the remaining two terms without interactions and it explained 61% of the variability observed in ratio of seeds produced.

For the remaining analysis, normality and homogeneity of the data was checked using the Shapiro-Wilk Test and Leven´s Test in R. If the data met both assumptions, it was followed up with a pairwise Student's t-test. For the remaining data, if the data did not meet the criteria for normality and homogeneity then the non-parametric Kruskal-Wallis test was initially performed, followed by a post-hoc Dunn´s test. P-values were adjusted using the Bonferroni method. Data correlations were done using the non-parametric Kendall correlation method. Categorical variables (e.g. subgenome) were compared using Pearson's χ^2 test. All statistical analysis was performed in R v.4.0.2.

4.4 Results

4.4.1 Unexpected genome instability in *Brassica* parental species

Five plants from each of seven different control genotypes from distinct established *Brassica* species (*B. napus* N1 and N5*; B. carinata* C1 and C2; *B. juncea* C1 and C2; *B. oleracea* O1) were analyzed for fixed (present in both homologous chromosomes) and segregating (present in one homologous chromosome) CNV events. Both events which were fixed per genotype across all five plants and events which were present only in one or a subset of plants per genotype were observed (Figure 1). Fixed genomic rearrangements were present in both *B. napus* genotype N1 and *B. carinata* C1 (Figure 1). Segregating events were also detected in genotypes *B. napus* N1 and N5, and *B. carinata* C1. Based on homoeology, duplication events observed in *B. napus* N1 are likely non-reciprocal translocation events between chromosomes A01/C01, A04/C04, C09/A10, and C09/A09. Three events involved an extra copy of the C genome replacing a region in the A genome, and two events involved an extra copy of the A genome replacing a region in the C genome. Loss of a whole chromosome was observed in three different plants (*B. napus* N1 and N5), affecting chromosome number (Figure 1, Figure 2).

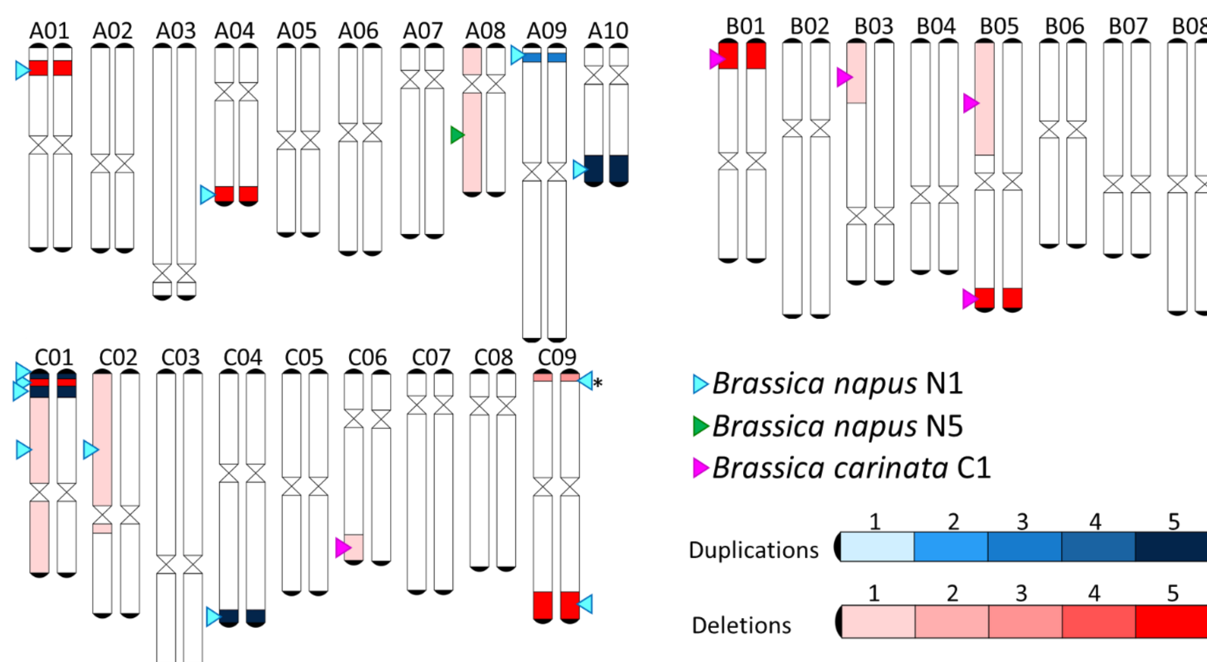


Figure 1. Combined molecular karyotype of *Brassica* species genotypes. Fixed (present in both homologs) and segregating events (present in one homolog) are represented. Frequency (in five assessed plants per genotype) is depicted in the legend. The genotype in which the rearrangement is present is indicated with an arrow head. *Deletion present as fixed in three plants and segregating in two plants.

4.4.2 Chromosome number in *Brassica* hexaploids

Nine different genotypes from four different allohexaploids types were selected: NCJ genotypes N1C1.J1, N1C2.J1, N6C2.J2, N5C2.J1a, and N5C2.J1b; carirapa genotypes C05, C13, C2128; and junleracea genotype J3O1. Out of the 218 allohexaploid plants analyzed, 20 had the expected chromosome number of 2n = 54, but only seven were true euploids with the correct numbers of chromosomes in each subgenome. The N1C1.J1 lineage contained the majority of the euploid plants (71%); N1C1.J1 line 2 from this genotype had 3/5 individuals with a euploid chromosome number, although with fixed and segregating chromosome rearrangement events (Figure 3). Different CNVs affecting chromosome number were also identified, with 0 to 4 copies present for each chromosome (Figure 2).

4.4.3 Chromosome number variants in the NCJ allohexaploid type

In the NCJ genotypes, 114 events involving the loss of both chromosome copies, 194 events involving one missing copy, 73 events involving an extra chromosome copy, and two events involving two extra copies of a chromosome were identified. In the NCJ genotypes, most of the events involving zero copies of a chromosome were present in the B genome, as seen in genotype N5C2.J2a for chromosomes B04 (all individuals with 0 copies), B05 (22 individuals with 0 copies and three individuals with one copy) and in genotype N6C2.J2 for chromosome B07 (20 individuals with 0 copies) (Figure 2). Single-copy chromosomes were frequently observed, especially for chromosome A02 (21 events) affecting mostly genotypes N5C2.J2b (seven plants from different lines), N6C2.J2 and N1C2.J1 (six plants each from different lines); and for chromosome C01 (19 events, mostly in genotype N5C2.J2b - 9 plants from different lines). Extra copy chromosomes in NCJ types were also observed particularly for chromosomes A01 and C02 (10 events in total for each) in genotype N5C2.J2b (in eight plants from different lines). Two extra copies of a chromosome were only observed in two instances in the NCJ allohexaploids, with one event each involving chromosomes A02 and C02 respectively, in one plant each of genotype N5C2.J2b lines 1 and 5. For both of these events the corresponding homeologous chromosome had zero copies (centromere was deleted) but the remaining regions of the chromosome were still present and translocated into the two extra copies (Figure 2).

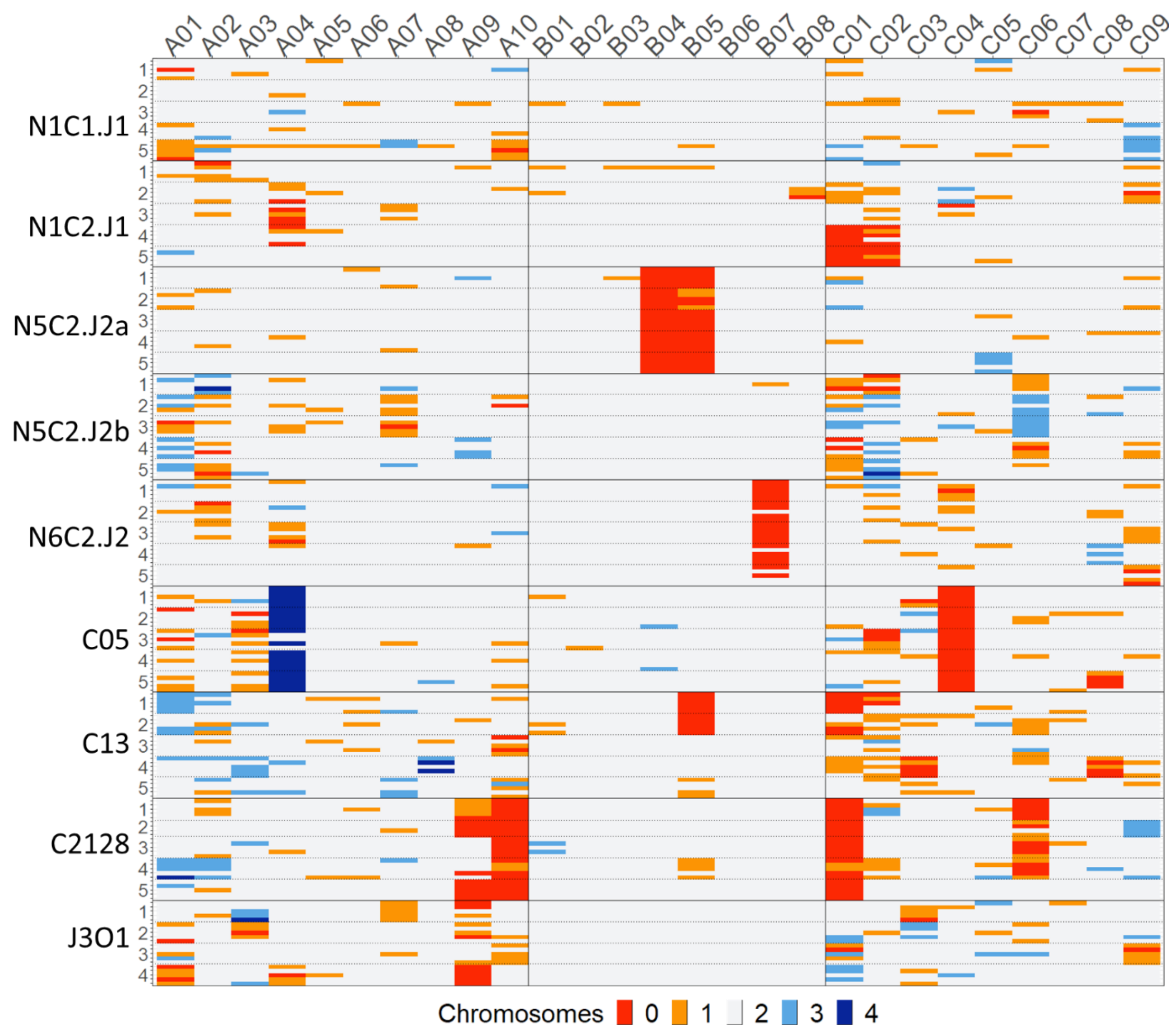


Figure 2. Chromosome copy number variants (CNVs) in different *Brassica* allohexaploid types. Genotype codes are written on the y-axis and within the graph they are marked by a horizontal line. Sibling lines are represented by numbers (1-4 or 1-5) and are marked within the graph by a horizontal dotted line. Within each sibling line 4-5 plants were analyzed, each represented by a line. The number of chromosomes present is colored according to the legend (0 = red, 1 = orange, 2 = light gray, 3 = pale blue and 4 = dark blue) for all chromosomes in subgenomes A (A01 - A10), B (B01 - B08), and C (C01 - C09).

4.4.4 Chromosome number variants in the carirapa allohexaploid types

In the carirapa genotypes, there were 131 completely absent chromosomes (zero copies), 137 instances of one chromosome copy, 60 instances of three chromosome copies, and 28 instances of four chromosome copies. Here, unlike the NCJ type, most of the chromosomes with zero copies were located in the C subgenome: in chromosomes C01 (29 observations) with the majority of these in only one genotype C2128 (22 out of 24 individuals analyzed), and in chromosome C04 (25 observations), present in all the plants analyzed from the genotype C05 (Figure 2). Interestingly, the homoeologous chromosome of C04, chromosome A04, was fixed and doubled in 22 of the 25 plants and the remaining region of C04 was translocated into the two extra copies of the chromosome (bottom 12.3 Mb of the chromosome). Single-copy missing chromosomes in carirapa types were observed in the highest number for chromosome C02 (19 observations) present in different individuals from genotypes C05 (four plants), C13 (10 plants), and C2128 (five plants), while an extra chromosome copy was found mostly for chromosome A01 in genotype C13 (eight plants), followed by A02 in genotypes C13 (six plants) and C2128 (four plants).

4.4.4.1 Chromosome number variants in the junleracea allohexaploid type

Junleracea genotype J3O1 had overall fewer chromosome CNV events compared to NCJ and carirapa allohexaploids, with only 16 observations of zero chromosome copies, 45 observations of one chromosome copy, 18 observations of three chromosome copies, and only one observation of two extra chromosome copies (chromosome A03). Most of the zero copy observations were present for chromosome A09 in lines 1 (three plants) and 4 (five plants), while single copy chromosomes were more evenly distributed between different chromosomes. Three copies of a chromosome were mostly observed for chromosome C01 in lines 2 (two plants), 3 (one plant), and 4 (three plants).

4.4.4.2 Genomic stability ranking between allohexaploid types based on segregating chromosome variation

Despite an overall high frequency of karyotype changes, some lines showed little chromosome variation across the five plants analyzed. In order to assess if karyotype change and hence genomic instability was still ongoing, segregating events were counted, since these events are more likely to indicate novel events, and hence may indicate if the karyotype has stabilized or not. Based on this metric, the 44 lines analyzed were ranked in terms of segregating CNV events (Supplementary Figure 1). From 1 up to 28 events per line were identified. Based on this analysis, the most stable genotype was N5C2.J2a line 3, which had only one single missing chromosome event involving C05 in one plant, although chromosomes B04 and B05 were completely lost in this genotype (fixed deletion/loss). The next most stable line based only on chromosome number changes was genotype N1C1.J1 line 2, which as mentioned previously was the line with the most euploid plants. This line also had only two segregating whole chromosome events present in two plants (one missing a copy of A04 and one missing a copy of C02). Carirapa C2128 line 5 also had few changes in single copy chromosomes, with only two plants affected: one with an extra chromosome A01 and one with a missing chromosome A02. This line had also completely lost chromosomes A09, A10, and C01. Interestingly, the line that had the highest number of segregating chromosome changes was also from carirapa C2128 line 4, with 28 events found in four of the five plants analyzed. The junleracea genotype was very unstable compared to many NCJ and carirapa lines, where the most stable line from this genotype was J3O1 line 4 with 14 single chromosome CNVs.

4.4.5 CNV and karyotype rearrangements in *Brassica* hexaploids

4.4.5.1 Fixed rearrangement events inherited from the parent allotetraploid species *B. napus, B. carinata*, and *B. juncea.*

We analyzed the total number of rearrangements in each of the *Brassica* allohexaploid lines per genotype. We identified pre-existing genomic rearrangements in *Brassica* parental genotypes N1, N5, and C1 (Figure 1). Also, from a previous study we identified rearrangements in the parental genotype N6 (deletion on chromosome C02, located between 8-10.7 Mb) (Quezada-Martinez *et al.*, 2022). To avoid bias when analyzing the number of CNVs present we looked at the corresponding allohexaploid produced by crosses of these genotypes, and only 84 fixed events (61 deletion and 23 duplications) were counted as directly inherited from the parents. These rearrangements were mostly present in genotype N1C1.J1, which had 46 events in total in the 24 plants analyzed (22 plants had a fixed deletion of the top of C01 and 24 plants had a fixed deletion of the bottom of C09), and in 15 individuals from genotype N1C2.J1 lines 1, 2, and 3 (14 plants with a fixed deletion of the top of C01 and one plant with a fixed deletion of the bottom of C09). The inherited fixed duplication events were only present in the allohexaploid genotype N1C1.J1, where the bottom of chromosome A10 was doubled and translocated into chromosome C09 in 23 of the 24 plants analyzed. We removed these inherited events from further analysis and kept only events characterized as new.

4.4.5.2 Genomic localization and distribution by allohexaploid type of fixed deletion and duplication events not involving changes in chromosome number

In total, we identified 1002 fixed deletion events and 664 fixed duplication events distributed between the allohexaploid types present on different chromosomes, where these events did not affect chromosome number. These events did not affect chromosome number because they were located in distal chromosome regions, i.e. not spanning or involving the centromere, and we used presence of the centromere as a proxy for chromosome number. The chromosome with the highest number of fixed deletions was C01, accounting for 12.9% of the

total, followed by chromosome A04 (9.2%), and chromosome A07 (9%). Six chromosomes (B01, B03, B04, B05, B06, and B08) did not show any fixed deletion events. The chromosome with the most fixed duplication events was C04, accounting for 13.5% of the total, followed by chromosomes C06 (10.8%), C02, and A01 (10.5%). Most of the chromosomes that did not have fixed duplication events were also from the B subgenome (B02, B03, B04, B07, B08), and one chromosome was from the C subgenome (C07). Most of the fixed deletion events were in a size range below or equal to 10 Mb (86.8%, Supplementary Figure 2) with an overall average size of 5 Mb and a minimum size of 1 Mb, the minimum size we were able to assess using this analysis method, and a maximum size of 26 Mb, equivalent to the loss of the bottom part of chromosome C07 in three individuals of carirapa allohexaploid genotype C13 line 5. Similarly, most of the fixed duplication events were ≤ 10 Mb (88.7%, Supplementary Figure 2) with an average of 5 Mb, a minimum size of 1 Mb and a maximum size of 35 Mb (a duplication at the top of chromosome C02 in one plant of N6C2.J2 line 3).

The size of fixed deletions and duplications differed significantly between allohexaploid types (Kruskal-Wallis test, p = 1.28e-19), with NCJ deletions being significantly larger (average of 7 Mb) than carirapa (Dunn´s test, p = 1.07e-19, average of 4 Mb) and junleracea (Dunn´s test, p = 1.38e-2, average of 5 Mb), and carirapa deletions being significantly smaller than junleracea type (Dunn´s test, p = 1.45e-3, Supplementary Figure 3). Fixed duplication size also differed significantly between allohexaploid types (Kruskal-Wallis test, p = 1.42e-6) with NCJ types showing significantly larger deletions than carirapa types (Dunn´s test, p = 6.85e-7, Supplementary Figure 3).

4.4.5.3 Genomic distribution of segregating CNV events in the allohexaploids

The number of segregating translocations was quantified in the *Brassica* allohexaploids. Eight events were removed based on parental inheritance: five missing copies of chromosomes A01, B01, and A04, and three duplications in C04: these were present in six plants of the genotype N1C1.J1. In total, 710 events involving the loss of a copy and 359 events involving the gain of a copy in the terminal regions of chromosomes were identified. Missing single copy events were evenly distributed between the A and C subgenomes, with 48.3% and 48.4% of the events, respectively, while the B genome had only 3.3% of the events. In the case of extra copies, the

A subgenome (45.5% of events) and C subgenome (60.7% of events) were not significantly different (Pearson's χ^2 test, $p = 0.137$), and the B subgenome had 1.4% of these events. C01 had the most missing regions, with 107 events (15.3%), followed by A01 with 70 events (10%), and in third position chromosome C02 with 61 events (8.7%). Chromosome C02 had the most duplication events, with 62 (17.3%), followed by chromosome A09 with 36 events (10%), and chromosome A01 with 35 events (9.7%).

4.4.5.4 Fixed deletion and duplication events not involving changes in chromosome number varied between lines within genotypes of NCJ, carirapa, and junleracea allohexaploid types

In NCJ allohexaploids, 268 fixed deletion events, 206 fixed duplication events, 288 missing copy events, and 194 extra copy events were identified. The different lines per genotype and the number of events per plant were compared to assess the effect of independent rearrangements segregating in the genotypes. Lines within the genotypes N5C2.J2a and N5C2.J2b had similar number of events per plant with no significant differences in any of the events analyzed (fixed deletions per plant, Kruskal-Wallis test, p = 0.406 and $p = 0.0846$; fixed duplications, Kruskal-Wallis test, $p = 0.118$ and $p = 0.0894$; missing copy, Kruskal-Wallis test, $p = 0.157$ and $p = 0.0536$; and extra copy, Kruskal-Wallis test, $p = 0.623$ and $p = 0.383$, respectively; Figure 3). Lines in the genotype N1C2.J1 only differed significantly in the number of duplication events per plant (Kruskal-Wallis test, $p = 0.00757$). Lines in the genotype N6C2.J2 only differed significantly in the number of fixed events per plant: deletions (Kruskal-Wallis test, $p = 6.5e-4$) and duplications (Kruskal-Wallis test, $p = 5.4e-3$). In the genotype N1C1.J1 the sibling lines had more variation and significant differences were observed in three categories: deletions (Kruskal-Wallis test, $p = 0.0124$), duplications (Kruskal-Wallis test, $p = 0.00415$), and missing copy (Kruskal-Wallis test, $p = 0.0144$).

In carirapa allohexaploids, 620 fixed deletions and 387 fixed duplication events were identified. Lines in the carirapa genotype C05 showed no significant differences in any of the rearrangement types analyzed (deletions, Kruskal-Wallis test, $p = 0.0758$; duplications, Kruskal-Wallis test, $p = 0.188$; missing copy, Kruskal-Wallis test, $p = 0.594$; and extra copy, Kruskal-Wallis test, $p = 0.826$; Figure 3). Lines in the genotype C13 showed only significant differences in the

fixed events: deletions (Kruskal-Wallis test, $p = 4.7e-3$) and duplications (Kruskal-Wallis test, $p = 6.5e-3$). On the other hand, lines in the genotype C2128 varied significantly, with statistical differences found in all the rearrangement types analyzed: deletions (Kruskal-Wallis test, $p = 5.8e-3$), duplications (Kruskal-Wallis test, $p = 0.0365$), missing copy (Kruskal-Wallis test, $p = 0.0243$), and extra copy (Kruskal-Wallis test, $p = 0.0164$) events.

Finally, in the junleracea allohexaploids (single genotype J3O1) 114 fixed deletion events and 71 fixed duplication events were found. Significant differences between the lines were found for all CNV types: deletions (Kruskal-Wallis test, $p = 0.00101$), duplications (Kruskal-Wallis test, $p = 0.0013$), missing copy (Kruskal-Wallis test, $p = 3.1e-3$), and extra copy (Kruskal-Wallis test, $p = 0.0112$, Figure 3) events.

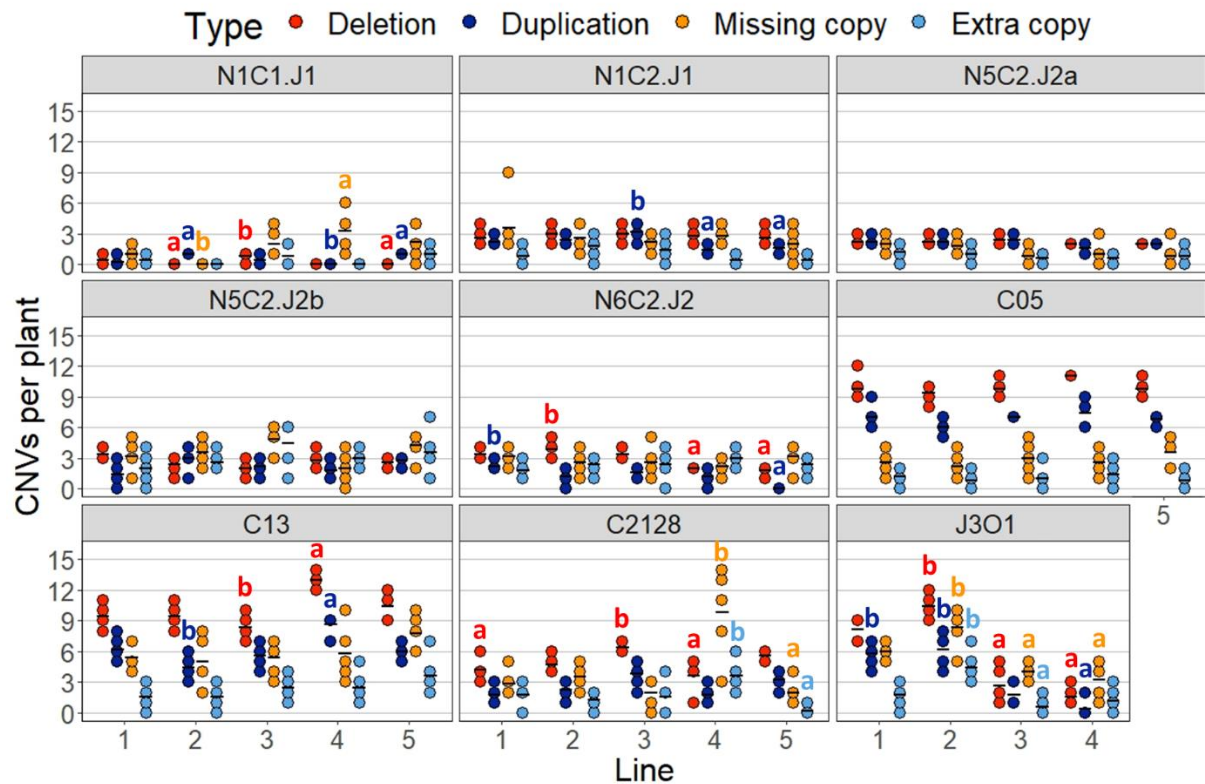


Figure 3. Copy number variants (CNVs) in *Brassica* allohexaploid lines grouped by genotypes. NCJ (*B. napus* × *B. carinata* × *B. juncea*) genotypes: N1C1.J1, N1C2.J1, N5C2.J2a, N5C2.J2b, and N6C2.J2; carirapa (*B. carinata* × *B. rapa*) genotypes: C05, C13, and C2128; junleracea (*B. oleracea* × *B. juncea*) genotype J3O1. Comparisons were made per CNV type between the lines of the same genotype. Each CNV type is colored according to the legend: red indicates deletions (zero copies), dark blue indicates duplications (four copies), orange indicates a missing copy (one copy) and pale blue indicates an extra copy (three copies) of each chromosome region. Mean values per line are shown as a horizontal black line. Statistically significant differences between the lines are shown with letters (the same color of letter represents the same CNV type comparison), where different letters represent significant differences between the lines (Kruskal-Wallis test, followed by Dunn´s test, significance at $p < 0.05$).

4.4.5.5 Duplication events without an identifiable genomic location for the duplicated region

For some single copy duplication events the specific location in the genome could not be determined, either because there was no missing copy in the respective homeologous region or because no missing copy in the rest of the genome was scored according to the parameters (> 1 Mb in size). In the NCJ allohexaploids, 26 duplications of A-genome chromosome regions (A01, A07, A09, and A10) with unknown chromosomal location were identified. Most of these events (19) involved a duplication of the bottom part of A10 (located between 18.4 – 19.9 Mb)

in genotype N6C2.J2 in different lines (line 1 - four plants, line 2 - five plants, line 4 - five plants, and line 5 - five plants). In the carirapa genotypes, only two single copy duplication events were identified of chromosome A09 regions with unknown locations, one located at the top of the chromosome (0 - 3 Mb) and one at the bottom of the chromosome (43 – 46.7 Mb), present in the same genotype of C2128 in line 4, but in two different plants. In the junleracea allohexaploids a single duplication event was found with an unknown location in chromosome A10, located between 0 - 1.9 Mb in a plant of line 2. Only one single copy duplication event with an unknown genomic location was observed in the B genome: this was in a carirapa plant from genotype C13 line 4, where B05 had a single copy duplication of the region from 13.8 – 23.4 Mb. For the C genome, 30 single copy duplication events were identified in NCJ allohexaploids, with most of them located on chromosome C02 (20 events), while the remaining duplicated regions involved chromosomes C04 and C09 (four events each), and C07 and C08 (one event each). In the carirapa type eight events were found, with three events located on chromosomes C01 and C03, and one single event for each of chromosomes C02 and C07. For the junleracea five duplication events were identified, with unknown genomic location: two events located on chromosome C05, and one event in each of chromosomes C01, C02, and C03.

4.4.5.6 Differences between genotypes in frequencies of different types of CNVs and correlations with selfing-rounds

Overall, significant differences were observed between genotypes for all CNV types analyzed: deletions (Kruskal-Wallis test, p = 1.29e-31), duplications (Kruskal-Wallis test, p = 1.71e-26), missing copy (Kruskal-Wallis test, p = 1.58e-15), and extra copy (Kruskal-Wallis test, p = 3.67e-12) (Figure 4). Genotype N1C1.J1 had significantly less deletions compared to the other genotypes (Dunn´s test, $p < 0.05$, Figure 4).

Since we have different generations, we were interested if there was any correlation between the total number of CNVs and the allohexaploid genotype generation: we identified a weak correlation between the two values (r = 0.3, p = 2.9e-09), where the older generations have more CNVs, as expected. On the other hand, when analyzed separately per allohexaploid type, the NCJ allohexaploids showed a significant negative correlation (r = -0.17, p = 0.021), and the

carirapa allohexaploids showed a significant positive correlation in number of CNVs relative to number of generations (r = 0.28, p = 0.0019).

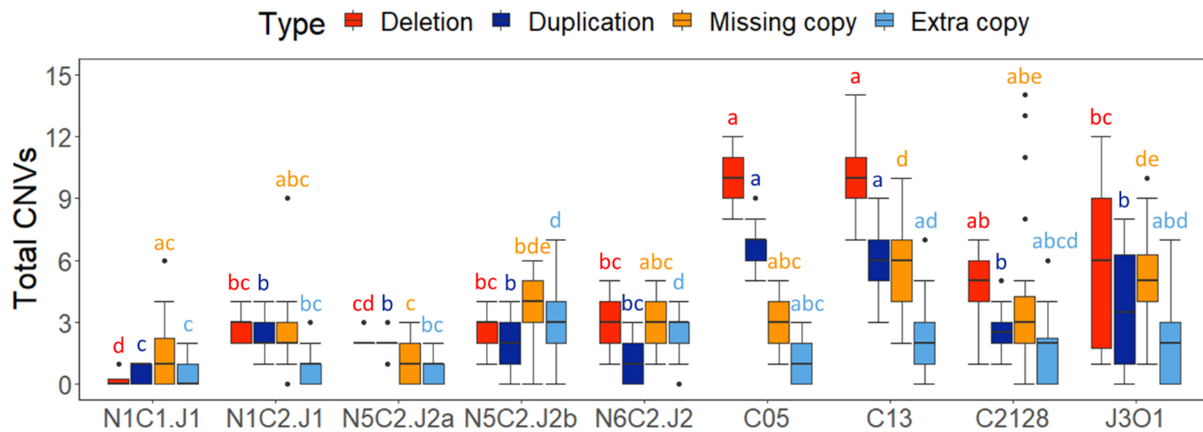


Figure 4. Copy number variants (CNVs) per genotype in different *Brassica* allohexaploids. NCJ genotypes: N1C1.J1, N1C2.J1, N5C2.J2a, N5C2.J2b, and N6C2.J2; carirapa genotypes: C05, C13, and C2128; junleracea genotype J3O1. Comparisons were made per type of CNV between all the allohexaploid genotypes. Each CNV type is colored according to the legend: red indicates deletions (zero copies), dark blue indicates duplications (four copies), orange indicates a missing copy (one copy) and pale blue indicates an extra copy (three copies) of each chromosome region. Statistical differences between the genotypes are shown with letters (the same color of letter represents the same CNV type comparison), where different letters represent significant differences between the genotypes (Kruskal-Wallis test, followed by Dunn´s test, significance at $p < 0.05$).

4.4.6 Effects of genotype and allohexaploid type on total CNVs

Allohexaploid type on its own (NCJ, carirapa, or junleracea) was able to explain 50% of the variation observed in total CNV numbers: carirapa, junleracea and NCJ allohexaploid type had effects of +24.3, +20.7, and +10.9 CNVs per plant (p = <2e-16, p = 0.0325, and p = <2e-16, respectively). Genotype-specific effects were also calculated in a linear model that was able to explain 70% of the variation observed in CNV accumulation, with all genotypes analyzed having a significant effect on total CNV accumulation (Supplementary Table 7). Significant effects of genotype on CNV totals ranged from +5.5 to +29.4 CNVs per plant. The smallest positive effects were found in genotypes N1C1.J1 (+5.5 CNVs, p = 4.3e-14), N5C2.J2a (+9.3 CNVs, p = 1.82e-4),

and N1C2.J1 (+11.5 CNVs, p = 3.85e-08), while the largest positive effect was observed for genotypes C13 (+24.9 CNVs, p = <2e-16), C05 (+24.9 CNVs, p = <2e-16), and J3O1 (+20.7 CNVs, p = <2e-16).

NCJ allohexaploids were a combination of a few genotypes from *B. napus* (N1, N5, and N6), *B. carinata* (C1 and C2), and *B. juncea* (J1 and J2). In the NCJ set we analyzed for the independent effect of these parental genotypes on the total number of CNVs. *Brassica napus* genotypes showed a positive effect on the total CNVs but only for two genotypes, with genotype N1 having the lowest effect of +8.5 total CNVs per plant (p = 1.21e-05), followed by N5 with +12.5 total CNVs per plant (p = <2e-16). *Brassica carinata* genotypes had more significant contrasting effects, with C1 having an effect of +5.5 (p = 1.48e-10), while C2 had an effect of +12.2 (p = 3.78e-12) total CNVs. Finally, *B. juncea* genotypes had a significant effect on total number of CNVs per plant of +8.5 (p = <2e-16) and +12.4 (p = 7.38e-06) for genotypes J1 and J2, respectively. Overall *B. napus, B. carinata,* and *B. juncea* on their own explained less than 35% of the variation observed (14%, 32%, and 15%, respectively).

4.4.7 Translocation events and subgenome bias in *Brassica* allohexaploids: fixed duplication/deletion events

For all fixed duplications not involving centromeric regions and hence most likely to comprise non-reciprocal translocation events, we were able to identify where in the genome the extra copies were located based on inspection of the primary homoeologous region. In total, we found that putative translocation events involving a C fragment replacing an A fragment (256 events) were significantly more common than an A fragment replacing a C fragment (372 events, respectively. Pearson's χ^2 test, p = 3.68e-06). Specifically, 12 different chromosome pairs were identified with fixed putative non-reciprocal translocations involving a duplication of the A genome translocated into the C genome (256 events of an A fragment into C genome chromosome). These events involved chromosomes A01 – C01 (10.5% of the total fixed non-reciprocal translocation events), A07 – C06 (9.6%), A09 – C08 (5.7%), A09 – C09 (4.5%), A03 – C03 (4.2%), A02 – C02 (2.4%), A10 – C09 (0.6%), A04 – C04 (0.3%), A05 – C04 (0.2%), A05 –

C05 (0.2%), A06 – C05 (0.2%), and A07 – C07 (0.2%). Twelve different chromosome pairs were also involved in a fixed translocation of a C genome fragment into an A genome chromosome (372 events): C04 – A04 (11.7% of the total fixed non-reciprocal translocation events), C06 – A07 (10.8%), C02 – A02 (10.5%), C03 – A03 (6.2%), C09 – A09 (4.2%), C05 – A05 (3.8%), C01 – A01 (2.6%), C04 – A05 (1.8%), C08 – A09 (1.7%), C05 – A06 (1.5%), C09 – A10 (1.1%) and C08 – A08 (0.2%).

The greatest number of putative translocations was observed between C04 and A04, where the C region was doubled and translocated into the A subgenome: these accounted for 78 events in total, with all these events involving the bottom portions of the chromosomes. At the same time, when the bottom of C04 was deleted (11 events), in only two events was the corresponding homoeologous region in A04 doubled and putatively translocated. For the B genome, only identified four different fixed putative non-reciprocal translocations were identified, all involving translocation of a B genome fragment into a C genome chromosome): B01 – C05 (3.6% of the total non-reciprocal translocation events), B05 – C01 (0.8%), B01 – C04 (0.6%), and B06 – C06 (0.5%). The size of the B genome fragment translocated varied, from 1.4 Mb (B05→C01 in two plants in C13 line 5) up to 17.6 Mb (B01→C04 in four plants of J3O1 line 1).

In the NCJ type, the number of A → C and C → A subgenome translocations were approximately the same (~50%), with no translocations involving B chromosomes. In carirapa allohexaploids, there were significantly more translocation events (59.9%, Pearson's χ^2 test, p = 2.12e-8) that involved a C chromosome segment replacing an A chromosome segment, while only 32.6% of events involved translocation of an A chromosome segment into the C genome, and 7.5% of events involved translocations from the B into the C genome. In the junleracea hexaploids, 47.9% of events involved translocation from the C to A genome, 42.3% involved translocation from the A to C genome, and 9.9% involved translocation from the B to C subgenome. Genotypes and lines within genotypes also varied in direction of fixed translocations between subgenomes (Figure 5).

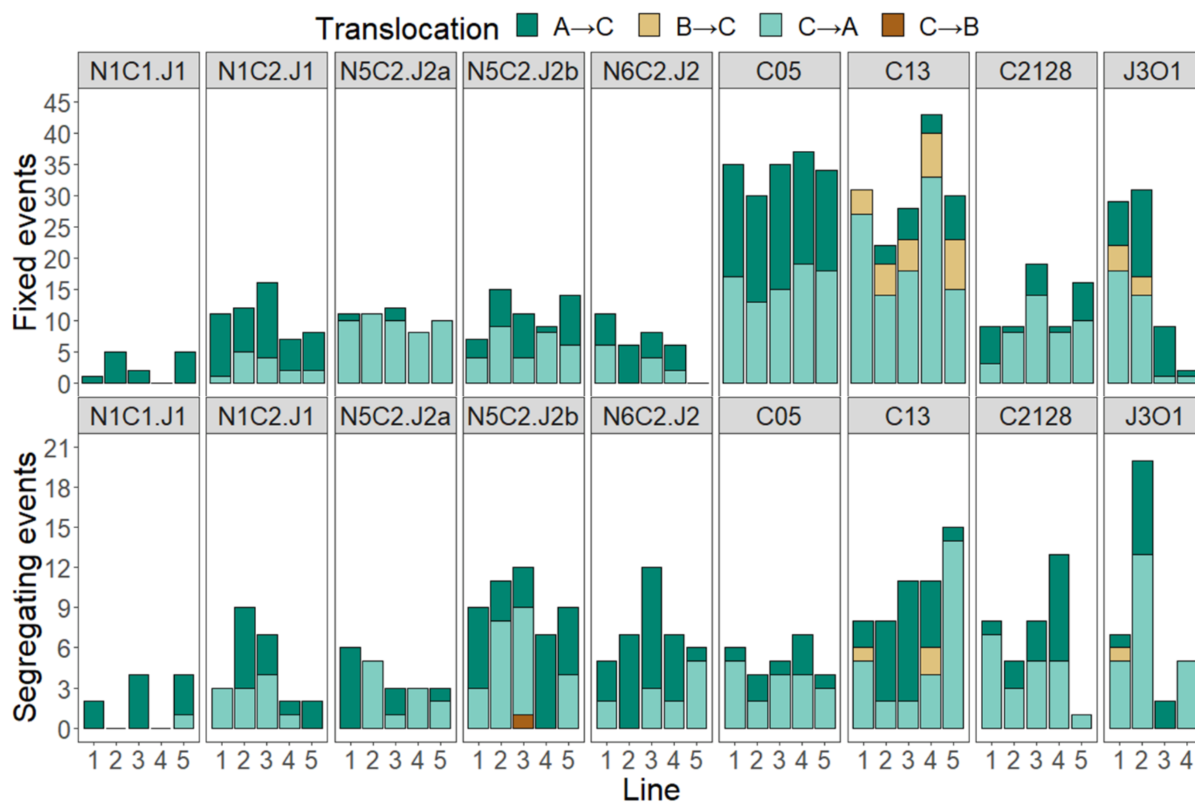


Figure 5. Translocations between the different *Brassica* subgenomes (A, B and C) in different allohexaploid genotypes. NCJ type (*B. napus* × *B. carinata* × *B. juncea*) genotypes: N1C1.J1, N1C2.J1, N5C2.J2a, N5C2.J2b, and N6C2.J2. Carirapa type (*B. carinata* × *B. rapa*) genotypes: C05, C13, and C2128. Junleracea type (*B. juncea* × *B. oleracea*) genotype J3O1. Genotype names are at the top of each rectangle. The upper graph represents the number of fixed translocations between the different subgenomes (see legend: e.g A → C = A-genome chromosome fragment duplicated and translocated into the C subgenome). Each bar represents one line per genotype (5 lines for the NCJ and carirapa allohexaploid types and 4 lines for the junleracea type).

4.4.8 Translocation events and subgenome bias in *Brassica* allohexaploids: segregating events (single copy / extra copy translocations)

A total of 286 single-copy (heterozygous, segregating) homoeologous translocation events between subgenomes were identified (Figure 5). The chromosomes commonly involved in non-reciprocal translocation events was similar between allohexaploid types, although the direction of the event differed, with no significant bias overall towards the direction of the translocation between the A and C subgenomes (Pearson's χ^2 test, $p = 0.438$). In the NCJ allohexaploid type, 138 events involving a putative translocation were found, where most of the events

corresponded to a duplicated A chromosome fragment translocated into a C-genome chromosome (57.2%), or to a duplicated C-genome chromosome fragment translocated into an A-genome chromosome (42%). Only one putative translocation involved the B genome, where a fragment of chromosome C04 was translocated into B06. In the carirapa allohexaploid type, most of the translocations occurred in the opposite direction from the NCJ, and involved a duplication of a C-genome chromosome fragment translocated into an A-genome chromosome (57.9%), while the A → C direction accounted for 39.5% of the events, and B chromosome fragments translocated into C-genome chromosome comprised 2.6% of the events. Finally, in the junleracea type, as in the carirapa type, most events involved translocation from the C subgenome into the A subgenome (67.6%): A → C events only accounted for 29.4% of the total, and one instance of a B-genome chromosome translocation into the C subgenome was observed between chromosomes B01 and C04.

4.4.9 Crossing *Brassica* allohexaploid genotypes

Flowering time varied between genotypes (Supplementary Table 2): the shortest flowering time was 32 DAS (one plant in genotype N4C2.J1), while the longest was 121 DAS (two plants in genotype N5C2.J2a), which affected the order in which plants were crossed. Genotype N5C2.J2a had the widest range of flowering time with an average of 95 ± 23 DAS, while the earliest flowering genotype N1C2.J1 had the least variation (average of 43 ± 1 DAS). Pollen viability for each plant was also estimated: 62% of the plants had < 50% viable pollen, and only 6% had pollen viability > 80% (Supplementary Figure 4). Two naponigra genotypes (N8.I1 and N9.I2) had poor anther development and produced no viable pollen (0%). Similarly, in the case of the N5.I2 naponigra genotype, pollen viability estimates could only be obtained from two of the four plants available.

In total, 113 different cross-combinations were carried out with 10 310 flower buds crossed. Out of the total crosses harvested, 48.7% (5023 crosses) developed into siliques, with a maximum success rate of 97.1% silique development in a cross between N7C1.J1 and N1C2.J1, where only 34 flower buds were crossed. However, most of the crosses produced a low ratio of seeds per bud pollination, ranging from 0.0 – 4.6 seeds/flower bud (Figure 6).

For some of the crosses, siliques were developed, but upon opening contained only shriveled seeds or no seeds at all. This was observed especially when N5.I2 was used as a male donor in the cross. We identified at least four cross combinations where 50% of the siliques developed after crossing, but very few seeds were obtained (a ratio lower than 0.3 seeds/bud pollination): genotype combinations N5C2.J2a × N5.I2, J3O1 × N5.I2, C2128 × N5.I2, and N5C2.J2b × N5.I2. The maximum number of seeds produced from a cross was obtained from N1C1.J1 × N5C2.J2a, where out of 109 flower buds crossed, 500 seeds were obtained (Ratio = 4.6, Figure 6).

Genotype affected the ratio of seeds produced per bud pollination (Supplementary Table 5). Genotype N1C1.J1 had a strong positive effect on the success ratio of seeds/bud pollination when used as female parent, adding an extra 1.0 seeds per bud pollination based on multiple regression analysis ($p = 0.00431$). Seven other genotypes showed a positive effect on ratio of seeds produced per bud pollination, ranging from 0.01 seeds/bud in genotypes N7C1.J1, up to 0.28 seeds/bud in genotype C05 (Supplementary Table 5). Genotype N9.I2 showed a negative effect as a female parent, with -0.32 effect in seeds/buds. Male parent genotype effect was only significant for one genotype (two lineages) with big effect of +1.82 and +1.95 observed for N5C2.J2a and N5C2.J2b. Together, female and male genotype combination was able to explain 47% of the variation observed in the ratio of seeds produced per cross combination (Supplementary Table 5, Figure 6).

Crossings between NCJ genotypes provided an excellent opportunity to analyze the effect of having a common *Brassica* parent genotype on both sides of the cross on the number of seeds produced per bud pollination (ratio, Supplementary Table 6). For example, in the cross combination N4C2.J1 × N5C2.J2a, *B. carinata* genotype “94024.2_02DH” (C2) is in common between the maternal and paternal parent line. Based on multiple linear regression analysis, having one ($p = 0.1642$) or two parents ($p = 0.0952$) in common had no significant effect on the ratio of seeds produced per bud pollination. Interestingly, having zero parents in common had a greater effect of +2.0 on seeds/bud pollination ($p = 0.0470$). Having three parents in common had a negative effect of -1.1, but only one cross combination represented this category ($p = 0.0108$). In the same model but considering only NCJ genotypes and including the female and male genotypes separately, only one genotype had a significant effect: genotype N5C2.J2a used as a female parent had a slight positive effect on ratio of seeds produced per bud pollination

(+0.6; $p = 0.0172$), and N5C2.J2b used as a male parent had a higher positive effect on ratio of seeds produced per bud pollination (+4.7, $p = 0.01388$).

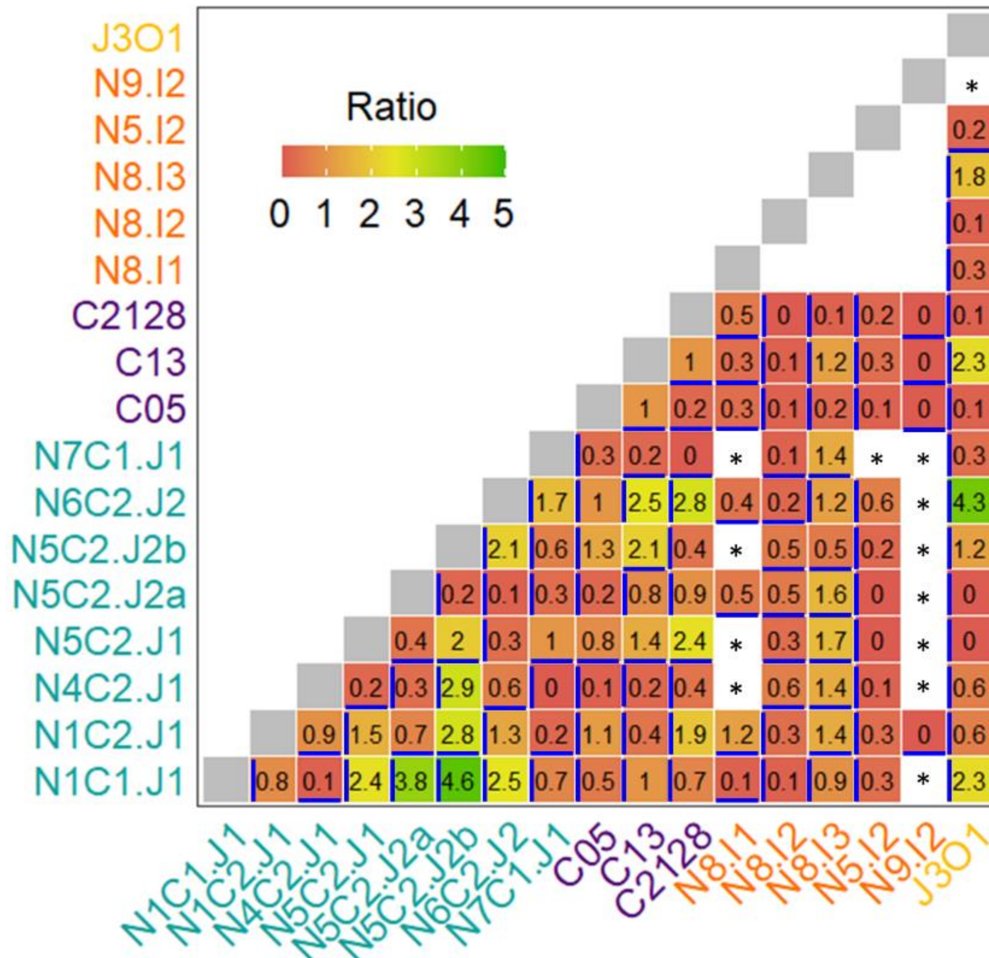


Figure 6. Crossing success between different allohexaploid *Brassica* genotypes. Ratio represents the total number of seeds produced divided by the number of flower buds cross-pollinated between genotypes. * Cross combination was planned but not performed due to experimental constraints. The female parent in the cross is marked with a perpendicular blue line relative to the genotype name: e.g. in N1C1.J1 × N1C2.J1, the female parent is N1C1.J1.

In total, 9052 new hybrid seeds were obtained from cross-pollinations between allohexaploids, out of which 835 had germinated in the silique at the time of harvesting. On average, 14.2% of the seeds were viviparous per cross combination. Maternal and paternal genotypes were compared in the number of viviparous seeds produced and significant differences were found only when comparing maternal genotypes used in the cross (Kruskal-Wallis test; $p = 5.7\text{e-}07$) and not when comparing male parent genotypes (Kruskal-Wallis test; $p = 0.3713$). Using a linear

regression model, four genotypes had a significant positive effect on the percentage of viviparous seeds produced: N7C1.J1 (+34.1%), N5C2.J2 (+31.5%), N4C2.J1 (+29.7%), and N1C1.J1 (+13%); however, the maternal genotype only explained 25% of variability observed (Supplementary Table 8).

The overall ratio of self-pollinated seeds produced per bud pollination was higher than the ratio obtained by crossing, ranging from 0.0 – 11.4 seeds/flower bud. Out of the 95 plants used for crossing, 17 were completely self-infertile, and genotype N9.I2 was completely sterile. Out of all the naponigra genotypes, seeds were only obtained from two plants of genotype N8.I2, where five harvested branches (secondary meristems) produced a total of eight seeds. Two of the five *Brassica* junleracea J3O1 genotypes were also self-infertile, together with one plant each of genotypes N7C1.J1 and N4C2.J1.

Total seed number and pollen viability were weakly correlated (r = 0.19, p = 0.0095), as were ratio of self-pollinated seeds per bud pollination and pollen viability (r = 0.25, p = 0.00069) and ratio of self-pollinated seeds per bud pollination and flowering time (r = 0.25, p = 0.0017). A stronger correlation was observed between number of developed siliques and total number of seeds obtained (r = 0.7, p = <2.2-16).

4.4.10 F_1 hybrids compared to the parents:

Eight F_1 hybrids were selected based on the number of seeds obtained and the different parental genotype combinations involved (Table 1). Unfortunately, self-pollinated seeds from one of the parents of the hybrids could not be obtained, genotype N8.I3, and hence this parent line is missing as a control to compare with the new F_1 hybrids. All the F_1 hybrids analyzed were true hybrids between the parents based on the genotyping data analysis.

Out of the eight F_1 hybrids, only three showed significant differences in flowering time compared to either of the parents (Supplementary Table 9). Hybrid C13 × J3O1 averaged 52 days to flower (DTF), significantly fewer than parent genotype C13-2 (average 78 DAS; Student's pairwise t-test, p = 0.0388), but not parent genotype J3O1-1 (average 45 DAS; Student's pairwise T-test, p = 1), although the parents did differ significantly in flowering time (Student's pairwise t-test, p = 0.0147). Similarly, the new F_1 hybrid N6C2.J2 × J3O1 flowered significantly

earlier (on average 51 DTF) than its parent N6C2.J2-1 (on average 69 DTF, Student's pairwise t-test, p = 0.0392), but no significant difference was observed when compared to the junleracea parent J3O1-1 (Student's pairwise t-test, p = 0.868); flowering time in DAS again differed significantly between the parents (Student's pairwise t-test, p = 0.0105). On the other hand, hybrid N8.I3 × J3O1 took significantly more DAS (72) to flower compared to its parent J3O1-2 (53 DAS; Student's t-test, p = 0.0323). All the other new hybrids showed similar DAS compared to their respective parents.

Four parental plants did not produce any seed in this new generation: J3O1-1, J3O1-2, C05-1, and N5.I2-1. The remaining plants produced between 1 – 3533 seeds (Supplementary Figure 5). Number of seeds produced by F_1 hybrids compared to their parents differed significantly in three of the cross combination analyzed (Figure 6). Only the F_1 hybrid C13 × C05 produced significantly more seeds than one of its parents (female parent genotype C13, Figure 6).

Table 1. Crosses between *Brassica* hexaploids NCJ (*B. napus* × *B. carinata* × *B. juncea*), junleracea (*B. juncea* × *B. oleracea*), carirapa (*B. carinata* × *B. rapa*), and naponigra (*B. napus* × *B. nigra*) genotypes selected for comparison with the parents. The individual parental plant used in the cross is represented by a hyphen followed by the number of the plant.

Genotype Female	Genotype Male	Hybrid Genotype	Flower buds crossed	Siliques developed	Seeds obtained	Ratio*
N1C1.J1-1	N6C2.J2-2	N1C1J1 × N6C2J2	99	58	251	2.5
N6C2.J2-1	C13-1	N6C2J2 × C13	110	62	277	2.5
N6C2.J2-1	J3O1-1	N6C2J2 × J3O1	44	41	191	4.3
N6C2.J2-3	N5.I2-1	N6C2J2 × N5I2	66	37	42	0.6
C13-2	J3O1-1	C13 × J3O1	100	79	231	2.3
C13-2	C05-1	C13 × C05	100	44	103	1.0
C13-3	N8.I3-1	C13 × N8I3	87	66	101	1.2
N8.I3-1	J3O1-2	N8I3 × J3O1	106	97	186	1.7

*****Seeds obtained per cross-pollinated flower bud

4.4.11 CNVs and seed set in parents and F_1 progeny

F_1 hybrids compared to both parents showed no correlation between total CNV present per plant and total number of self-pollinated seeds obtained (r = -0.046, p = 0.63). However, a correlation was observed between presence of an extra chromosome or extra fragment from

the A subgenome and total seed number (r = 0.24, p = 0.015). Similarly, the number of A-genome chromosomes present and the number of total self-pollinated seeds was also significantly correlated (r = 0.21, p = 0.031). All the plants analyzed in the F_1 were aneuploid (Supplementary Table 8), with chromosome number ranging from 43-57 chromosomes in total. Only one F_1 hybrid differed significantly from either of its parents in terms of number of CNVs present: N8I3.J3O1, where the F_1 hybrid had fewer CNVs compared to J3O1-2 (Supplementary Figure 6).

One dwarf sterile plant was observed in the parental genotype J3O1-1. During germination, this plant emerged with four cotyledons, and despite being able to develop and flower it did not phenotypically resemble the other sibling lines. This plant did not have a high number of CNVs (16 CNVs) and the majority of the rearrangements affected the number of chromosomes, and as a consequence only 43 chromosomes were present (Supplementary Table 8). Another sterile dwarf plant from the same allohexaploid combination was an individual from J3O1-2, with many rearrangements (39 CNVs) and 47 chromosomes present.

Interestingly, in genotype C05, which only had two plants available, a similar number of CNVs was observed between the plants (27 and 31), but the plant with a lower number of CNVs produced 2223 seeds compared to zero seeds produced by the hybrid with 31 CNV events. CNV events between these plants not only affected chromosome segments, but also chromosome number, where the hybrid that produced more seeds had 53 chromosomes (missing both copies of C04 and one copy of A10, but had 4 copies of A04), and the hybrid which produced zero seeds had 49 chromosomes (missing both copies of A01, A02, and C04, single copy of A07, A10, C07, C08, an extra copy of C01, C02, and C05, and two extra copies of A04). One naponigra plant N5.I2 also produced no seeds, had few CNVs (8) and 52 chromosomes with missing copies of A02, A09, C01 and C06, and an extra copy of C03 and C09. This plant also had no anther development and short filaments.

4.5 Discussion

In this study, we firstly aimed to systematically compare frequencies and types of CNV events observed between advanced allohexaploid *Brassica* lineages produced from different species and genotype combinations. Previous studies have suggested that differences exist between allohexaploid lineages (different species combinations) in terms of meiotic stability (Mwathi *et al.*, 2017) and genomic stability (Zhou *et al.*, 2016b), and genotype-specific effects have previously been established to arise from different parents in segregating mapping populations (Gaebelein *et al.*, 2019b), but here we undertake the first comparison across multiple allohexaploid types and genotype combinations, using high-resolution molecular karyotyping. We found strong effects of genotype and allohexaploid type on overall accumulation of CNVs per plant, with carirapa genotypes accumulating the most CNVs, followed by junleracea, then NCJ types. Each of *B. napus, B. juncea* and *B. carinata* parent genotype had a significant influence on total number of CNVs per plant, suggesting genetic factors inherited from each of these species affect non-homologous recombination frequency in the allohexaploids, as also suggested by (Gaebelein *et al.*, 2019b). Similarly, genotypic differences on chromosome pairing behavior (bivalent and univalent frequency during meiosis) have been observed in *Brassica* carirapa allohexaploids, with one parental *B. rapa* genotype identified to confer almost 100% bivalent formation (Gupta *et al.*, 2016). *Brassica* allohexaploids of four different species combinations were previously observed to show some cytological differences in pairing behavior and genomic stability with carirapa (S_7) having the highest, followed by junleracea S_{0-1}, *B. rapa* × *B. oleracea* × *B. nigra* with 44.4% (Zhou *et al.*, 2016b). Genotypic effects on the frequency of non-homologous recombination events have also been observed cytologically in *B. napus* (Sheidai *et al.*, 2003), in interspecific hybrids between *B. juncea* and *B. napus* (genome composition AABC; (Mason *et al.*, 2010)), and using molecular marker segregation methods in interspecific hybrids between *B. napus* and *B. carinata* (Mason *et al.*, 2011). In synthetic *B. napus* genotypes, parental *B. oleracea* and *B. rapa* genotype combinations also affected number of CNVs present in progeny after one generation of self-pollination (two meioses) (Katche *et al.*, 2023). Our results suggest that many genetic variants inherited from parent species and genotypes play a role in meiotic and genomic stability in allohexaploids, and highlight the importance of starting with a broad genetic base in establishment of a new allohexaploid *Brassica* crop.

Crosses between *Brassica* allohexaploid types have never before been systematically attempted, and we were curious to see if these behaved as “interspecific” crosses due to the different species parents, or as “intraspecific” crosses due to the shared combination of A, B and C genomes. Pre- and post-fertilization barriers were found to be active in *Brassica* allohexaploid crosses, as many siliques failed to develop and very few seeds were obtained from a number of cross combinations. Many of the plants used in the crosses had viable pollen, but only approximately half of the crosses resulted in developed siliques. In previous studies of diverse crosses between *Brassica* species and ploidy levels, the main barrier to producing new hybrid seeds was the failure of pollen to fertilize the ovule (Nishiyama *et al.*, 1991b), and in our crosses, a similar phenomenon might have hindered success in the crosses. The average number of self-seeds produced per silique was higher compared to the values obtained from crossing, also suggesting some incompatibilities when trying to cross between different allohexaploid types with diverse species origin. Out of the five naponigra genotypes, we only obtained self-pollinated seeds from genotype N8.I2. However, we also identified genotypes of *Brassica* naponigra allohexaploids that were more successful at crossing than at producing self-seeds. The differences between self-pollinated and cross-pollinated seed production were especially evident for genotype N8.I3, a genotype that was used as a female and male parent in different crosses but which produced no self-pollinated seeds from three cutting-derived plants (with three selfing bags per plant). In previous studies of the same *Brassica* naponigra genotypes, irregular meiosis was suggested as the main cause of low seed set, while self-incompatibility (present in the *B. nigra* parent species) was thought to have played a smaller role (Gaebelein *et al.*, 2019a). In our study, self-incompatibility seems to have stronger importance, since the same plant was able to produce over 100 seeds from crossing, but under self-pollination conditions not a single seed was developed. We also identified genotypes with significant maternal and paternal influence on number of seeds produced per bud pollination in our crossing scheme. Maternal effects have also been observed in crosses between *B. napus* and *B. rapa,* where the cross has a greater success when *B. napus* is used as a female parent (Fitzjohn *et al.*, 2007). Similarly, when recreating *B. napus* synthetics*,* maternal *B. rapa* parent genotype strongly influenced the success of the interspecific cross with *B. oleracea* (Diederichsen & Sacristan, 1994; Lu *et al.*, 2001; Abel *et al.*, 2005).

Interestingly, 3/7 of our “control” *Brassica* genotypes, representing established allotetraploid species, also contained CNV events. These events were not observed in all five plants analyzed

per genotype combination, and some CNVs affected only one of the homologous chromosomes present, suggesting ongoing genomic changes and instability in natural *Brassica* allotetraploids. In support of our results, non-homologous translocations between the A and C genomes have previously been observed in *B. napus* using various methods, including microscopy (Osborn *et al.*, 2003; Sheidai *et al.*, 2003) segregation distortion of mapping populations (Schranz & Osborn, 2000; Stein *et al.*, 2017) , and sequence read mapping depth (Chalhoub *et al.*, 2014; Samans *et al.*, 2017). Non-homologous pairing in polyploids has also been observed in several other polyploid species, although the frequency is rather low compared to synthetic polyploids (Madlung *et al.*, 2005; Henry *et al.*, 2014; Ihnien Katche *et al.*, 2022) and, even though different chromosomes might interact during the initial stages of meiosis, these configurations tend to resolve into correct homolog pairing (bivalent formation) as meiosis progresses (Comai *et al.*, 2003). How meiotic stabilization or diploid-like behavior has been achieved in natural polyploids is not yet fully understood, although many potential hypothesis have been described, involving for example new mutations, changes in genetic regulation, or inheritance of pre-adapted alleles (reviewed in (Gonzalo, 2022)). To the best of our knowledge, our study is the first to report genomic instability in a *B. carinata* genotype: this was unexpected due to the wider genetic divergence between the *Brassica* B and C genomes relative to the A and C genomes (Parkin et al. 1995, Lagercrantz and Lydiate 1996, Perumal et al. 2020). It is possible that this finding relates to the fact that this *B. carinata* genotype is derived from microspore culture to produce doubled-haploid lines, which may confer genomic instability (Shrestha *et al.*, 2023). However, as both *B. carinata* genotypes used in this study are derived from the same microspore culture process (also at the same time point with the same protocol), there may also be variation for non-homologous chromosome recombination frequency within genotypes of the parent allotetraploid species, as has been hypothesized on the basis of cytological results for *B. napus* genotypes (Sheidai *et al.*, 2003). Possibly, such non-homologous rearrangements in the allotetraploid parent species frequently occur but are usually removed due to selection pressure, rather than accumulating in parent lines (Gaeta & Pires, 2010).

Brassica allohexaploids showed a propensity to reduce genome size. This was observed by the higher number of deletions and missing copy events present in the allohexaploids compared to duplications and extra copies, affecting both whole-chromosomes and chromosome-fragments. Similar trends have also been observed in other allohexaploid studies, such as in carirapa H_2 lines, and in three NCJ allohexaploid populations, where the majority of the

individuals lost chromosomes in subsequent generations (Tian *et al.*, 2010; Gaebelein *et al.*, 2019b). Cultivated *Brassica napus* accessions also showed more deletion events compared to duplication events (Higgins *et al.*, 2018), suggesting that the reduction in genome size is not only an attribute of synthetic material but also may occur in "natural" genotypes. This reduction in genome size could also be an early sign (or ongoing process) of diploidization, similar to what is observed in natural polyploids, where duplicated regions are being lost and rearranged (Li *et al.*, 2021b). However, as seen in other studies (Stein *et al.*, 2017; Higgins *et al.*, 2018), duplication/extra copy events are harder to identify using genotyping data, and this might have also influenced our results: hence, more research is needed to see if the propensity to lose chromosomes and chromosome fragments is maintained in future generations.

Accumulation of CNVs from previous generations had a major effect on genome stability: we found different degrees of stability between sibling lines based on the number of accumulated CNVs (Figures 2 and 3). In other words, many lines which originated from the same genotype combination differed from each other in stability and number of accumulated CNVs. This was particularly true for genotype N1C1.J1. Genotype N1C1.J1 contained line 2, which was putatively the most stable line out of the whole set of allohexaploids analyzed: three out of five plants had the expected number of chromosomes (2n = 54), and the other two plants lost only one copy of chromosome A04 or C02, respectively. At the same time, line 2 of N1C1.J1 only had one new CNV event while the remaining events present corresponded to events previously identified in the parent *B. napus* "N1" genotype and hence likely inherited directly from the parent into the allohexaploid. Since the majority of the new CNVs found in N1C1.J1 line 2 appear to be directly inherited from *B. napus,* it is possible that these rearrangements played a beneficial role in stabilizing the karyotype of the new allohexaploids. However, no specific translocation was found that uniquely differentiated "stable" from "unstable" lines within this genotype combination. Interestingly, in synthetic *B. napus* a deleted region in chromosome C01 from 2.5 – 8.3 Mb was previously identified to be associated with lower numbers of seeds produced per flower (Ferreira de Carvalho *et al.*, 2021), although in our case the deleted region in both N1C1.J1 and *B. napus* "N1" was a bit smaller (from 2.2 - 3.3 Mb on chromosome C01), and did not seem to affect seed production.

An overall bias in translocation direction between the A and C subgenomes was observed for fixed non-reciprocal translocation events, where significantly more instances were found

where a C-genome fragment replaced an A-genome fragment, compared to where an A-genome fragment replaced a C-genome fragment. However, at the genotype level, there were different patterns of bias in either A → C or C → A translocations, with differences also found between lines within genotypes. For example, genotype N1C1.J1 (containing the putatively stable line 2) had only fixed translocation events where an A fragment replaced a C fragment, while genotype N5C2.J2a had more fixed events where a C fragment replaced an A fragment as a result of non-homologous recombination. In natural and synthetic *Brassica napus* an opposite trend has been observed, with the A genome replacing the C genome more frequently than the C genome replaced the A (Samans *et al.*, 2017; Higgins *et al.*, 2018). Interestingly, translocations in allohexaploids where an A fragment replaced a C fragment have previously been associated with a positive effect on fertility (Gaebelein *et al.*, 2019b); this effect was previously found for genotype N5C2.J2. For this same genotype in our study (N5C2.J2a), we observed the opposite of this translocation bias effect to the later generations (more C fragments translocated into A chromosomes), although we selected for high-fertility genotypes in our study.

Four different fixed putative non-reciprocal translocations between a B-genome chromosome and a C-genome chromosome were found, B genome introgressions obtained in *B. napus* were also found to be more common in the C genome compared to the A genome (Dhaliwal *et al.*, 2017); in this study, 17 out of 23 B-genome segments identified were introgressed into the C subgenome: eight involved chromosomes B06 or B07, while the remainder could not be identified to the chromosome level. In interspecific hybrids between *B. napus* × *B. carinata* followed by two rounds of backcrossing, there was only one indication of a B-chromosome segment introgression involving a portion of chromosome B05 which introgressed into either chromosome A01 or C01, while the remaining B chromosomes were either missing or present as whole additional chromosomes (Navabi *et al.*, 2011). In our study, only one event involving translocation of an A genome fragment into the B subgenome was found. However, care should be taken about generalizations related to frequency of C vs. A-genome introgressions of B-genome segments, due to the low numbers of these events observed in our study.

We also observed the apparent loss of B-chromosome fragments or whole chromosomes without evidence for an accompanying duplication/translocation event. B genome chromosome loss was primarily observed for three chromosomes: B04 (NCJ genotypes), B05

(NCJ and carirapa genotypes), and B07 (NCJ genotype). NCJ allohexaploid types were produced via a two-step crossing process, whereby some loss of univalent A- and B-genome chromosomes from the *B. napus* × *B. carinata* (CCAB hybrid) likely occurred (Mason *et al.*, 2010, 2012) . However, whether there is specific selection against these three B chromosomes in particular (especially B07, which was also lost in carirapa lines) is unknown. Navabi et al. (2011) also found loss of whole B chromosomes and deletions in terminal regions (Navabi *et al.*, 2011), which may suggest preferential loss of these chromosomes and translocation regions, although more data is needed to confirm this result.

Despite intensive selection for several generations, most of the allohexaploid plants analyzed were aneuploids, and we were only able to identify seven plants with the expected number of chromosomes. Most likely, this can be attributed to the means of selection in each generation, which was fertility (number of seeds produced) rather than euploidy. Although fertility is known to be correlated with regularity of meiotic behavior in allohexaploids (Gaebelein *et al.*, 2019b), many allohexaploids have also been observed to show high fertility despite presence of large number of CNVs (Mason *et al.*, 2014b, 2015). In previous studies in *B. napus* synthetics, the number of euploid plants increased per generation upon successive selection of euploid individuals for several generations, with initial generations S_1-S_3 having 88.5 - 88.7% euploid plants, and later generations (S_8) having 100% euploid plants (Ferreira de Carvalho *et al.*, 2021). *Brassica napus* synthetics that are not under generational selective pressure show no evidence of karyotype stabilization, and tend to accumulate extra chromosomes (Xiong *et al.*, 2011). We did not observe accumulation of extra chromosomes in the allohexaploid lines, but it might be possible that we underestimated the number of duplication events using our SNP genotyping method. In another study of a subset of wheat synthetic hexaploid lines, aneuploidy was observed as a characteristic of synthetic nascent hexaploid lines and, despite selection for euploidy in early and subsequent generations, karyotype stabilization and reduction of aneuploidy was not achieved (Zhang *et al.*, 2013). In *Tragopogon* allopolyploids, aneuploidy is also still frequently observed after approximately 40 generations (Chester *et al.*, 2012, 2015), suggesting pressure to stabilize meiosis may also not always be present in natural systems. In early generations (H_2) a large set of *Brassica* carirapa allohexaploids produced from crossing several different accessions (29 *B. rapa* and 107 *B. carinata*) showed high levels of aneuploidy (91%) and low levels of putative euploid plants (4.6%) (Tian *et al.*, 2010). Although euploidy was

rare in our study, it is possible that more stringent selection in each generation for euploid chromosome complements might improve generational stability.

Viviparous seeds were observed in most of the crosses we made. We also identified significant differences in the number of viviparous seeds obtained based on the maternal genotype used in the cross. Similarly, in previous studies of crosses involving *B. napus* and *B. rapa,* viviparous seeds were also obtained (4.2%), but only when *B. rapa* was used as the female parent (Hauser & Østergåurd, 2004; Jenkins *et al.*, 2005). In previous studies it was proposed that the germination of seeds inside the silique could be a response to incompatibility between the new hybrid seed (maternal and paternal genomes) and its new silique environment (determined by the maternal genome) (Hauser & Østergåurd, 2004). Although the exact reason why viviparous seeds occurred so commonly in our crossings requires further study, it is important to account for a potential loss of hybrid seeds due to this premature germination, which also makes the resulting seeds more prone to drought, fungal infection or simply death (Jenkins *et al.*, 2005).

Out of the eight new selected F_1 hybrids, only the hybrid C13b × C05 produced significantly more seeds (heterosis) than both parents, possibly because C13 and C05 were both originally 100% homozygous genotypes (in contrast to NCJ types, which are heterozygous in the first generation). In the case of the hybrid N6C2.J2a × J3O1a, despite having no significant differences in total number of CNV between parents and hybrids, the parent N6C2.J2a produced significantly more seeds than parent J3O1a and the respective hybrid. We also observed that in a cross between N8.I3 × J3O1, the F_1 hybrids had significantly lower total numbers of CNVs compared to the parent J3O1. In previous studies of F_1 hybrids between carirapa and NCJ type allohexaploids, new F_1 hybrids accumulated and produced more novel rearrangements compared to the parents despite potential disadvantages in meiosis (Quezada-Martinez *et al.*, 2022). The lack of heterosis in our F_1 hybrid study is not surprising, since it was been observed that the main factor affecting fertility (seed number) in *Brassica* allohexaploids is the presence of absence of rearrangements (Gaebelein *et al.*, 2019b).

4.6 Conclusions

Our results suggest that genomically stable synthetic *Brassica* allohexaploids are achievable, but only at extremely low frequencies, and that stability may not always be under positive selective pressure due to the unpredictable relationship between fertility and genome composition in these hybrid types. Combining different allohexaploid types via hand-pollination is feasible, allowing new allelic combinations to be produced. However, the majority of the F_1 hybrids analyzed in the present study showed no significant improvement in fertility or genome stability compared to their parents. Translocations between the A-B and C-B subgenomes were also observed, and may be valuable for use in introgression breeding programs.

5. General discussion

In this thesis I investigated genomic stability, chromosome inheritance, seed fertility, and crossability between and within four *Brassica* allohexaploid types (2n = AABBCC = 54): naponigra (*B. napus* × *B. nigra*), carirapa (*B. carinata* × *B. rapa*), junleracea (*B. juncea* × *B. oleracea*), and NCJ (*B. napus* × *B. carinata* × *B. juncea*). Using SNP genotyping data, I was able to identify copy number variants (CNVs) present in different genomic regions and use this information to estimate genomic stability as the central topic of this thesis. I was also able to identify different CNV types (deletion, missing copy, duplication, and extra copy), their specific chromosome location, size, frequency, and putative translocations (Chapters 3 and 4). Analyzing the extent of genome rearrangements provided valuable information to understand the consequences of polyploidization and potential ways in which stabilization might be achieved in *Brassica* allohexaploids. Of particular interest for researchers is the information found in Chapters 3 and 4, related to non-homologous translocations between B/C and B/A chromosomes. B subgenome species (e.g. *B. nigra*) are a rich source of resistances genes (reviewed in Chapter 2) and as such, they could be implemented in breeding programs aiming to introgress new resistance into the narrow genetic diversity present in *B. napus* cultivars. Understanding which B chromosomes are more likely to recombine and produce an introgression into A/C subgenomes might facilitate the design of improved breeding programs (Chapter 2) and allow a proper likelihood assessment of successfully obtaining the desire introgression/s.

In Chapter 4, I was able to identify positive and negative parental genotype effects that gave origin to NCJ allohexaploids with fewer CNVs. This information can be used to select future parental genotypes to produce new *Brassica* polyploids with high or low non-homologous recombination events. At the same time, the differences observed between allohexaploid types (Chapter 4) might be useful in understanding the effect of different *Brassica* species combinations and how to address different challenges in the establishment of *Brassica* allohexaploid as a new crop.

For the first time, multiple hand crosses were attempted and achieved to combine between and within different allohexaploid types (Chapters 3 and 4), however, with different degrees of success. More than 9000 new F_1 allohexaploid hybrids seeds were produced (Chapter 4), and

these seeds could be use in future projects related to QTL analysis for fertility and genome stability, testing agronomic potential, novel oil profiles, heterosis, and to allow further selection of promising genotypes to continue working on the establishment of *Brassica* allohexaploid as a new crop, among other uses.

Trigenomic *Brassica* allohexaploids (2n = AABBCC) do not exist in nature but they can be produced via human intervention (Chen *et al.*, 2011b; Gaebelein & Mason, 2018). Unlike natural polyploids, new synthetic polyploids are often genomically unstable due to incorrect chromosome pairing during meiosis (Comai, 2005). How meiosis can become stable in neopolyploids is not yet fully understood but several possible routes have been put forward, such as spontaneous mutation, selection of particular existing variation (e.g. found in the ancestors), genome fractionation events, changes in epigenetic regulation, or introgression of pre-adaptive alleles (reviewed in (Gonzalo, 2022)).

In Chapter 4, the genetic stability of nine different allohexaploid lineages was analyzed. The majority of the allohexaploids were found to have unstable genomes, with the exception of N1C1.J1 line 2. Unfortunately, we were unable to identify a specific rearrangement that could be attributed to the stability observed. Several QTLs have been identified to be associated with genome stability and fertility in *Brassica* synthetic material (e.g. (Gaebelein *et al.*, 2019b; Higgins *et al.*, 2021; Katche *et al.*, 2023)) however none of these overlapped with any of the rearrangements found in our stable material. Meiotic stabilization in our material could also be achieved due to other consequences of polyploidization that we were unable to detect using genotyping data, such as transposon activation (Madlung *et al.*, 2005), methylation and gene expression changes (Lukens *et al.*, 2006; Zhang *et al.*, 2015b), inversions (Mandáková *et al.*, 2010), rearrangement events smaller than 1 Mb, reciprocal translocations (Xie *et al.*, 2010), or a combination of multiple other factors, therefore further research and follow up is require to understand from where is the stability is coming from in the N1C1.J1 line 2.

In Chapter 4, we found that the parental genotype also played a role in the genomic stabilization of allohexaploids, particularly of the NCJ type. Depending on the *B. napus, B. carinata,* or *B. juncea* genotype used in the cross, it had had a different effect in the total number of CNVs. However, in this particular case and due to the way in which the allohexaploids were produced, a particular allele can have two possible contributors: e.g. the A subgenome alleles can be inherited from *B. napus* or *B. juncea*. Because of this, further

identification of allelic variants and biases for a particular parental allele can further elucidate where the stability might come from in this material. Several QTLs with different parental allele contributions that affect fertility and meiotic stability have previously been identified in NCJ allohexaploids (Gaebelein *et al.*, 2019b). These QTLs were found to be mainly inherited from *B. carinata* or *B. juncea* genotypes, and several QTL had underlying meiotic candidate genes (Gaebelein *et al.*, 2019b). Similarly, in synthetic *B. napus* material, allelic variants of meiotic genes inherited from *B. oleracea* parents were associated with genome stability (total CNVs) (Katche *et al.*, 2023) supporting the idea that homoeologous recombination depends on the genotype used as a parent.

In Chapters 2 and 4 we identified rearrangements present in some of the parents of the *Brassica* allohexaploids that were unknown to us. As discussed above, parental genotype has been shown to have a significant effect in the genomic stability of the resultant synthetic polyploid. Due to this parental effect, it would be advantageous to test parents (particularly tetraploids and higher ploidies) for their genomic stability prior using them as parent. However, this does not guarantee stable progeny, as it was seen in Chapter 4. In Chapter 4, despite being produced from the same parental combination and crossing event, sibling lines (one lineage) exhibited different stability, indicating an ongoing segregation for meiotic stability factors in these lines despite having been self-fertilized for at least four generations. Similarly, in the early generation analysis of N5C2.J2a and N5C2.J2b genotype allohexaploids (in the publication referred to as “G1” and “G2” genotypes), different lineages from the same genotype combination differ in the total number of chromosomes (Mwathi *et al.*, 2017), illustrating that genomic stability is much more complex than choosing the right parental combination.

In Chapter 3, we observed that new F_1 allohexaploid hybrids are less meiotically stable (more CNVs) compared to the parents, contrary to what we initially hypothesized. Comparing the overall *de novo* rearrangements, the F_1 hybrids produced a similar number of new CNVs compared to the NCJ parents, but significantly more rearrangements than the carirapa parents. In chapter 4, we tested a new set of F_1 hybrids and we analyzed the performance (seed fertility) and CNVs present in the different F_1s and, similarly to what we observed in Chapter 3, they did not perform better than the parents. These results are not surprising, since the F_1s are accumulating different CNVs inherited from the parents, and these rearrangements are known to negatively influence fertility in synthetic and cultivated *Brassica* (Osborn *et al.*, 2003;

Gaebelein *et al.*, 2019b; Mwathi *et al.*, 2019; Katche *et al.*, 2023). These inherited CNVs present in the F_1 can also be the origin of new rearrangements (Udall *et al.*, 2005), working more as a cascade of genomic instability that produces more genomic instability after every generation, which might be the case of what we observed in Chapter 3. Supporting this idea, we found a significant positive correlation between the number of selfing rounds (generations) and the number of CNVs accumulated in the plant (Chapter 4). Despite this, we have only analyzed a subset of the F_1 hybrids, and different results might come from other genotype combinations, particularly crossings involving the putatively stable N1C1.J1 line 2 genotype as one of the parents (Chapter 4).

High numbers of non-homologous rearrangements were found in the allohexaploid genotypes using SNP genotyping data (Chapter 3 and 4). Chromosomal rearrangements are often seen in synthetic polyploids (Chen & Ni, 2006), but they can also occur in more recent natural polyploids like *Tragopogon* (Chester *et al.*, 2012, 2015) and *B. napus* (Higgins *et al.*, 2018). Meanwhile, other polyploids like rice (*Gossypium* spp.) and *Spartina* show little to no genomic changes after polyploidization (Liu *et al.*, 2002; Baumel *et al.*, 2002), suggesting that non-homologous recombination might work differently under natural conditions and that different taxa may have evolved separate mechanisms of stabilization. To be able to stablish a stable new *Brassica* allohexaploid as a crop type we need to ensure genomic stability (low number of new rearrangements) (Zhang *et al.*, 2021). However, our results showed that despite our efforts to select high-seed performance genotypes, allohexaploid lines are still undergoing genomic changes and many of the rearrangements are not being fixed in the genome (missing copy and extra copy) and, although there are regions more likely to undergo rearrangements, the patterns between lineages seems to be random. But what if we could avoid non-homologous recombination (meiosis) to produce a stable *Brassica* allohexaploid? Certain plants reproduce asexually via a process known as apomixis, producing clone seeds identical to the mother plant without undergoing fertilization and meiosis (Grimanelli *et al.*, 2001). For example, apomixis has been achieved in *Arabidopsis* and rice by the knock-down of meiotic genes (Marimuthu *et al.*, 2011; Khanday *et al.*, 2019), however its application in other crops remain to be tested. Nevertheless, plant breeding in general might benefit from bringing in apomictic synthetic systems into crops by not only allowing synthetic plants like *Brassica* allohexaploid to avoid meiotic errors but also as a tool of maintaining heterozygosity in F_1 hybrids (Mahlandt *et al.*, 2023).

Although non-homologous recombination events are an undesirable characteristic to establish allohexaploids as a crop, we could still take advantage of these events and use allohexaploid lines as an in-between-step to introgress traits of interest into elite cultivars (reviewed in (Chen *et al.*, 2011b)), particularly those found in the B subgenome (Chapter 2; (Gaebelein *et al.*, 2019a)). For example, *Brassica* allohexaploids have been used to improve traits in *B. napus* such as genetic diversity (Li *et al.*, 2006) or seed color (Wen *et al.*, 2012), and understanding the different frequencies of recombination between chromosomes is of major relevance to confidently design future experiments.

We used seed fertility as a proxy for genome stability and as a selection base for the genotypes used in this thesis (Chapters 3 and 4), although this indicator proved to not be stringent enough: many of the selected highly fertile genotypes had accumulated several CNVs and showed unstable genomes (chapters 3 and 4). In synthetic and cultivated *B. napus,* the total CNV events correlated negatively with the total number of seeds produced, supporting our selection criterion for stability (Samans *et al.*, 2017; Katche *et al.*, 2023). However, these correlations had a weak or not significant value in the allohexaploids, indicating the need for an extra selection step to ensure better candidate lines for genomic stability. In the current thesis, we have also complemented the phenotypic observations of putatively stable meiosis with pollen viability percentages (Chapter 4). Studies in allotetraploid *Arabidopsis* using pollen viability as a proxy for meiotic irregularities (aneuploidy) demonstrated a strong negative correlation, however no significant correlation was found between meiotic irregularities and seed number (Henry *et al.*, 2014). Studies in a *Brassica* allohexaploid mapping population showed no significant correlation between seed fertility and pollen viability (Yang *et al.*, 2016a) however no estimates were done for meiotic stability. At the same time, pollen viability can be heavily influenced by environmental conditions (Pacini & Dolferus, 2019) and many of the variations observed in our synthetic material could be the result of other issues independent of meiotic irregularities.

Another selection method for stable allohexaploids could be cytogenetics. Cytogenetics, despite being labor intensive and time consuming, represents a direct look at meiotic irregularities, including pairing behavior and segregating (Bennett, 2004), that ultimately are the origin of the different rearrangements observed in our plant material. Ultimately, we characterized stability based on the data from the SNP array (Chapter 3 and 4). In previous studies in synthetic *B. napus* populations, cytological observations (pairing behavior) had a high

correlation with non-homologous events obtained from the SNP array (Higgins *et al.*, 2021), strengthening the idea of analyzing future experiments using SNP genotyping as the main selection criterion for meiotic stability, and perhaps adding cytogenic observations to confirm stability.

We were able to generate molecular karyotyping for all of our allohexaploid plants using SNP genotyping data from the *Brassica* 90K Illumina SNP genotyping array (Chapter 3 and 4). Our focus was on CNV events, defined as a gain (duplications or extra copy) or loss (missing copy or deletions) of chromosome segments larger than 1 kb (Zmieńko *et al.*, 2014), although in our case we use ≥ 1 Mb in size as selection criteria due to limitations of SNP density on the array. Overall, we identified more CNV events involved in the loss of at least one copy than those events related to the gain of at least an extra copy. As seen before in other studies, deletion events are easier to detect in a genotyping data output (such as GenomeStudio), as we see them as lack of signal or "no call" (NC) marker (Rousseau-Gueutin *et al.*, 2017; Mason *et al.*, 2017; Stein *et al.*, 2017; Higgins *et al.*, 2018). On the other hand, duplication events are harder to spot, since we rely on the Log R ratio values (normalized signal intensity for each SNP in the array) (de Araújo Lima & Wang, 2017). Log R ratio has its limitations when it comes to duplication events, because it is not possible to accurately determine more than three copies due to signal saturation. Also, Log R ratios values can be influenced by, for example, different batch reads (Zhao *et al.*, 2018) or DNA quantity (Diskin *et al.*, 2008), making it more difficult to accurately identify a duplication event. Several bioinformatic pipelines have been designed to calculate CNV events, however the results contain inaccuracies and often require more scrutiny post initial analysis (Wineinger & Tiwari, 2012). In the present thesis, CNV scoring was done manually, by reviewing each SNP marker (allele and Log R ratio values), characterizing the event depending on the type (e.g. deletions or duplication) followed by identifying potential homoeologous exchanges between subgenomes. This method produces more accurate results (particularly true for duplication events) although the time spent analyzing the data is considerably larger (Zhao *et al.*, 2018). In future experiments it would be important to stablish a faster way to analyze the CNV data without compromising quality of the results, or at least reducing the man-hours spent.

The production of new interspecific hybrids between *Brassica* species, wild relatives, and/or allies can be challenging due to different pre and post-fertilization barriers (Stebbins, 1958). Creating a new *Brassica* allohexaploid crop type relies on interspecific crosses to be able to combine the three subgenomes (A, B, and C), hence, the successful production of trigenomic hybrids can be affected by these fertilization barriers. For example, production of carirapa allohexaploids (*B. carinata* × *B. rapa*) has fair success, with an average of 0 – 1.2 seeds per hand pollination (Gaebelein & Mason, 2018), while junleracea (*B. juncea* × *B. oleracea*) allohexaploid production has a lower success with an average of 0.8 seeds per pollinated bud or even in some cases, complete seed abortion (Weerakoon, 2011). In the case of NCJ allohexaploids (*B. napus* × *B. carinata* × *B. juncea*), the hybrid success is a bit more complex, since it involves a two-step cross, with the initial cross producing on average 1.12 seeds per bud pollinated, and the second step is far less successful with an average of 0.12 seeds per bud pollinated (Mason *et al.*, 2012). At the same time, production of carirapa and junleracea allohexaploids involves an extra step of chromosome doubling using colchicine treatment, adding a new variable to the success rate in hybrid production, reducing the number of final allohexaploids obtained per crossing. Due to this, the overall amount of available allohexaploid genotypes is limited, restricting genetic diversity and our capacity to select for potential beneficial alleles.

In this thesis, in Chapter 4, I was able to cross between four different allohexaploid types. Under natural conditions, interspecific hybridization happens rarely (Bing *et al.*, 1996) but we were able to show that the production of hybrids between allohexaploid types it is possible and that the hybrids obtained from the crosses are fertile. We were able to obtain thousands of new hybrid seeds from the crossings, involving the combination of the six species from the U´s triangle (U, 1935). Thanks to these cross combinations, we rescued alleles from parents that were self-sterile (particularly naponigra), that otherwise would have been lost due to self-incompatibility.

We brought new genetic diversity into our material by crossing different genotypes and species of allohexaploids (Chapter 3 and 4) even though there is already new diversity being produced thanks to the CNV events. CNV events have been previously associated with phenotypic changes in crops (reviewed in e.g. (Zmieńko *et al.*, 2014; Gabur *et al.*, 2019)) affecting traits in *Brassica* related to seed quality (Stein *et al.*, 2017), flowering time (Schiessl *et al.*, 2017), and disease resistance (Gabur *et al.*, 2018). In our case, the only potential trait affected by CNV that

we evaluated was genomic instability. However, it is still unclear the specific role of CNV and meiotic stabilization in *Brassica* allohexaploids. Despite this, there is more research needed to analyze the agronomic potential and probable future use of *Brassica* species as a new crop type.

6. Future work

In the current thesis we obtained relevant new results related to the genomic stability in *Brassica* allohexaploids. However, there are still many unanswered questions such as: where does the genomic stability come from in the genotype N1C1.J1 line 2? An important next step would be to confirm this genetic stability using cytogenetics. Meiosis configuration analysis and chromosome counting could be performed in already available samples. Furthermore, genomic stability should be also tested in further generations to determine if the stability is maintained and passed to new generations. Moreover, analysis comparing stable and unstable genotypes, focusing on meiotic genes (meiocyte gene expression) might allow the identification of putative candidate genes related to genomic stability and potential effects of hybridization and polyploidization in *Brassica* allohexaploids. At the same time, in the present thesis I analyzed our material using SNP array data that has its limitations. Future studies involving *Brassica* allohexaploids might benefit from utilizing other technologies to detect CNVs and other genomic changes such as next generation sequencing (e.g. long reads like PacBio). This technology has become more accessible with the advantage of providing more detailed information compared to SNP array and might allow a better understanding of the genomic consequences after polyploidization and hybridization in *Brassica* synthetics.

I produced diverse hybrid material including crosses between stable and unstable *Brassica* allohexaploid lines. This material could be used to generate mapping populations to identify QTLs related to meiotic stability and fertility in these lines. Also, and more towards evolutionary biology, some of this material could be used to generate a natural selection and hybridization process as a natural population, similar to what was done in *Tragopogon* (Chester *et al.*, 2012). In here, different allohexaploid genotype combinations could be grown under natural conditions for several generations. After, the karyotype and allele retention would be analyzed. This experiment might provide information regarding allele bias (with particular look into meiotic genes) related to the establishment of *Brassica* allohexaploids.

We identified parental genotype effects based on the *Brassica* species used. It would be valuable to identify new *Brassica* parental genotypes that produce progenies with reduced level of genomic rearrangement. This can be done by creating novel *Brassica* allohexaploid material

using other *Brassica* genotypes than the ones used in this study and doing a similar study to this thesis.

Further investigation outside meiotic instability in *Brassica* allohexaploid could also be carried out. Somatic instability (mitotic instability) is rarely analyzed in synthetic material, and very little information is available up to now. More research into this could be relevant into how much polyploidization and hybridization processes affect different cell processes.

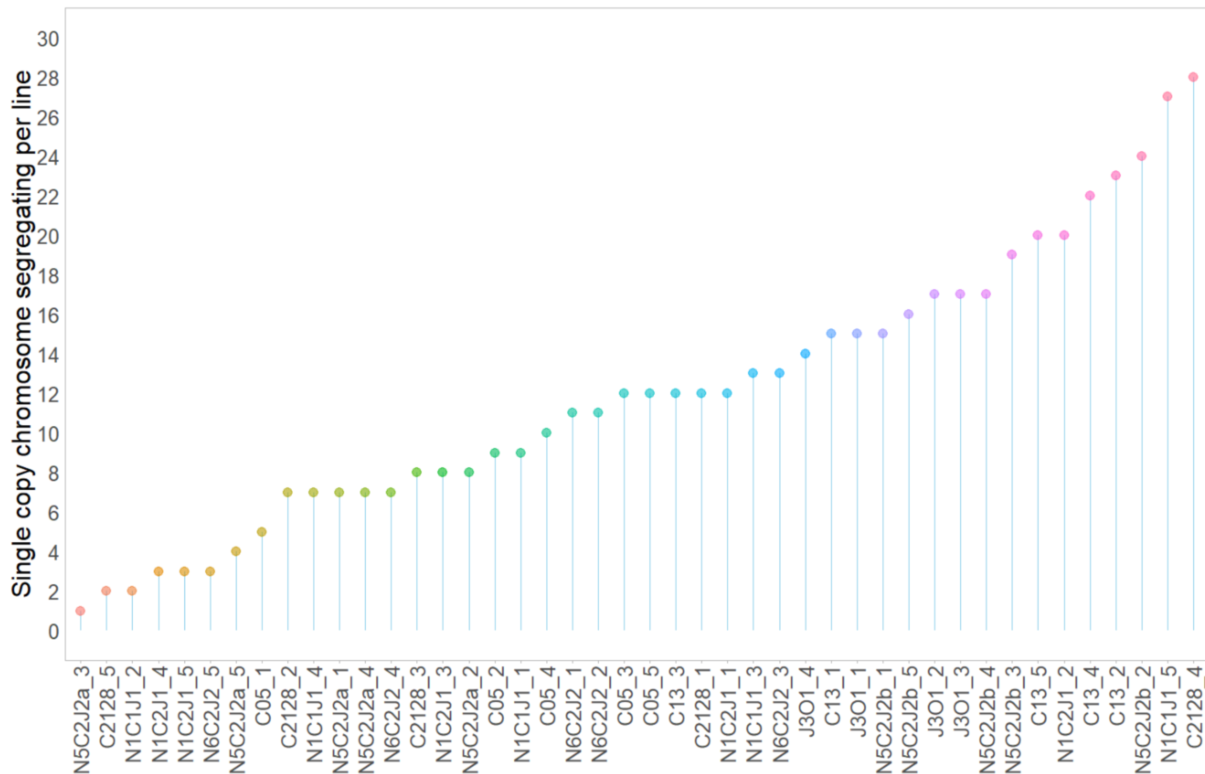
Finally, the agronomic potential of *Brassica* allohexaploids still remains to be tested. Many new phenotypes could be obtained from the different allohexaploid types and allele combinations including, but not limited to oil profiles, vegetable or ornamental type, disease resistance traits, growing type, flower color, etc. Field test and greenhouse experiments could be performed to analyze transgressive phenotypes and the potential for agricultural purposes, ideally applied to putatively stable material.

7. Conclusion

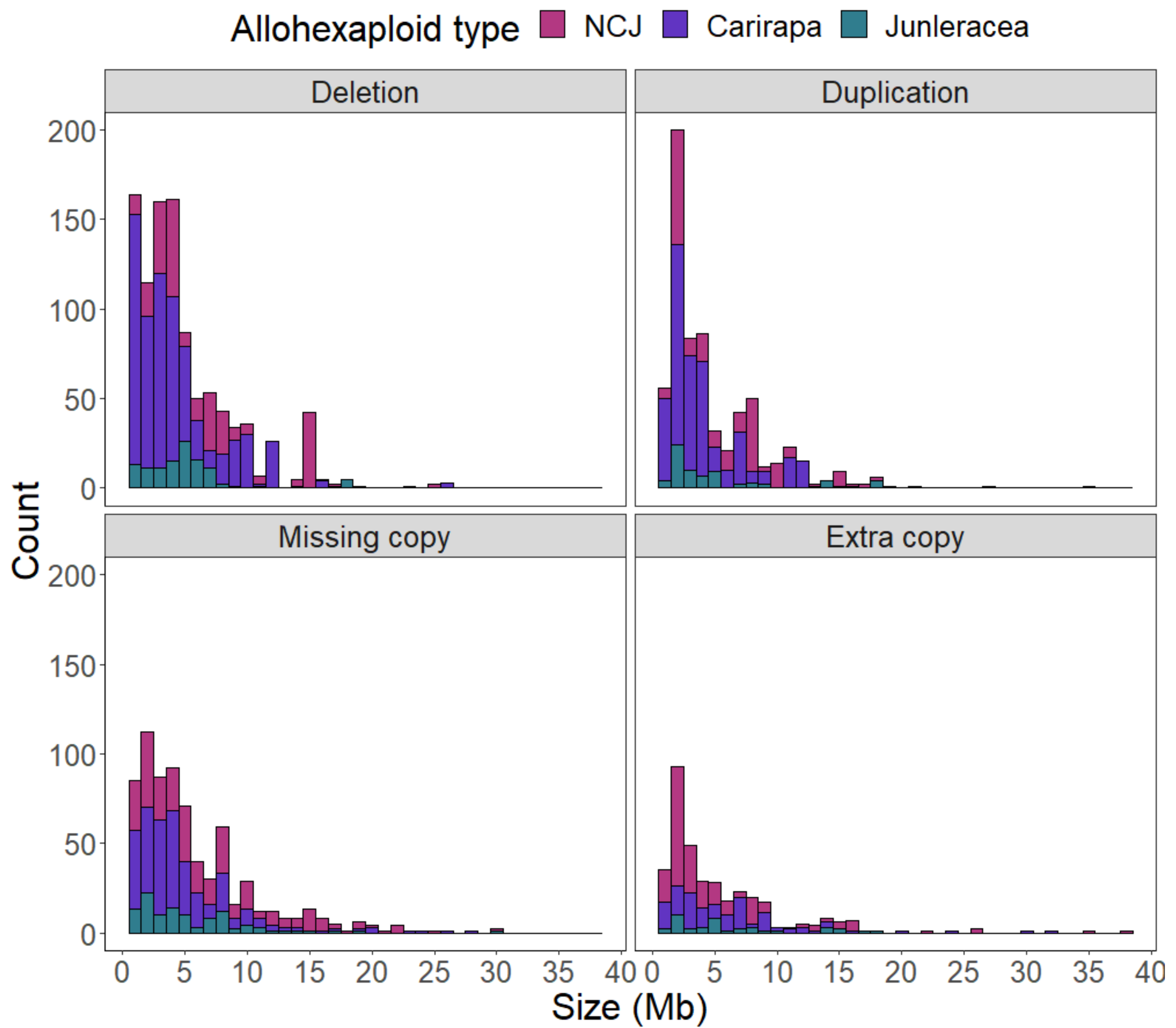
The results found in this thesis show that *Brassica* allohexaploid types are highly tolerant to CNVs and that their genomes are still undergoing genomic changes. We did not find a common pattern of rearrangements between different lineages, although certain genomic regions were more prone to undergo non-homologous recombination events. Putative genomic stability was only found at low frequencies and particularly in one line. Additionally, genomic stability may not be subject to positive selection pressure in *Brassica* allohexaploids, as the relationship between fertility and genomic stability is uncertain. We also demonstrated that allohexaploid types can be combine via hand-pollinations and that hybrids seeds can be obtained at different ratios. This allowed us to create a new set of allelic combinations, helping us expand the genetic diversity of the available material. However, the majority of the F_1 hybrids analyzed did not perform better than the parents: no significant improvement in the number of self-seeds produced or reduction in the number of CNVs. Despite this, the identification of a putatively stable *Brassica* allohexaploid line opens a new research opportunity to investigate further how this line became stable and if the stability is maintained in further generations.

8. Appendix

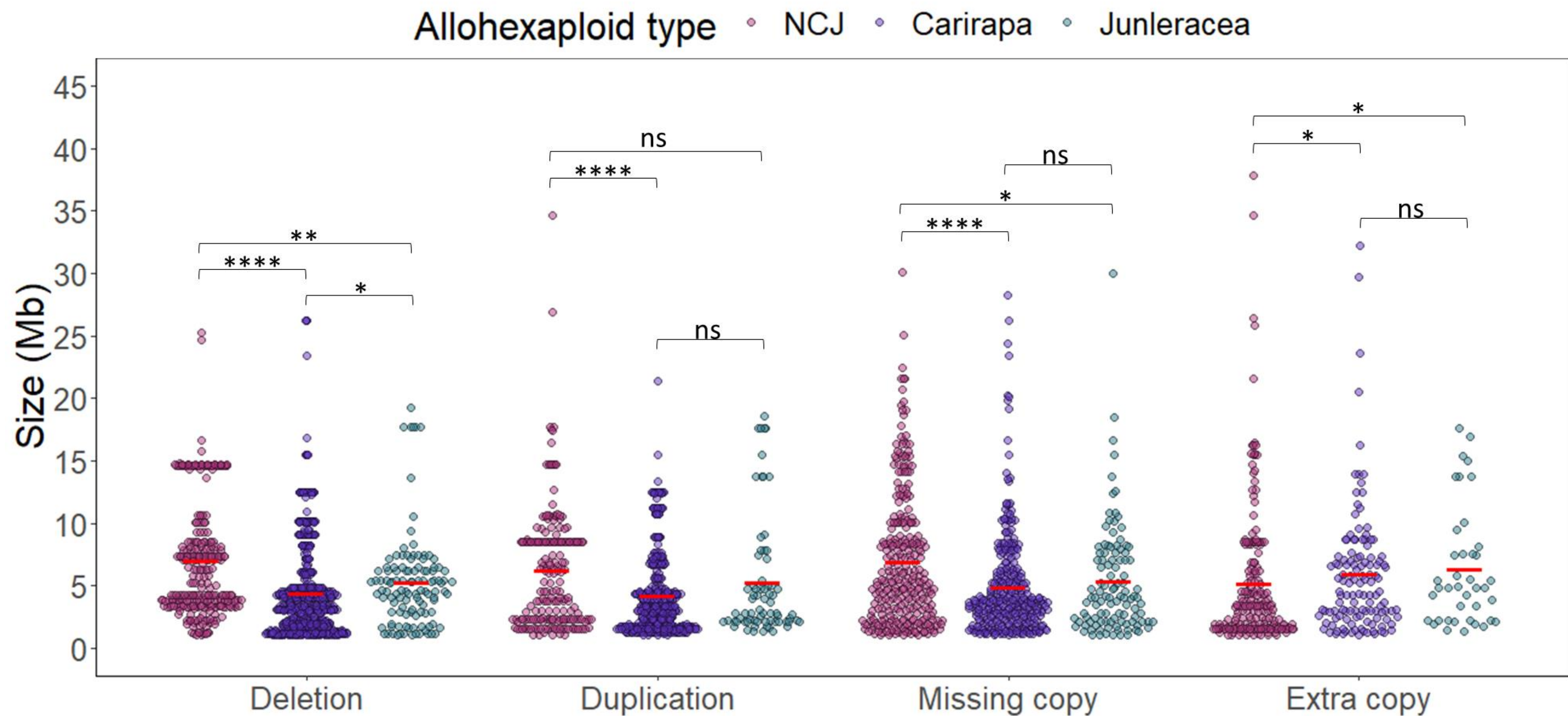
8.1 Supplementary Figures and Tables



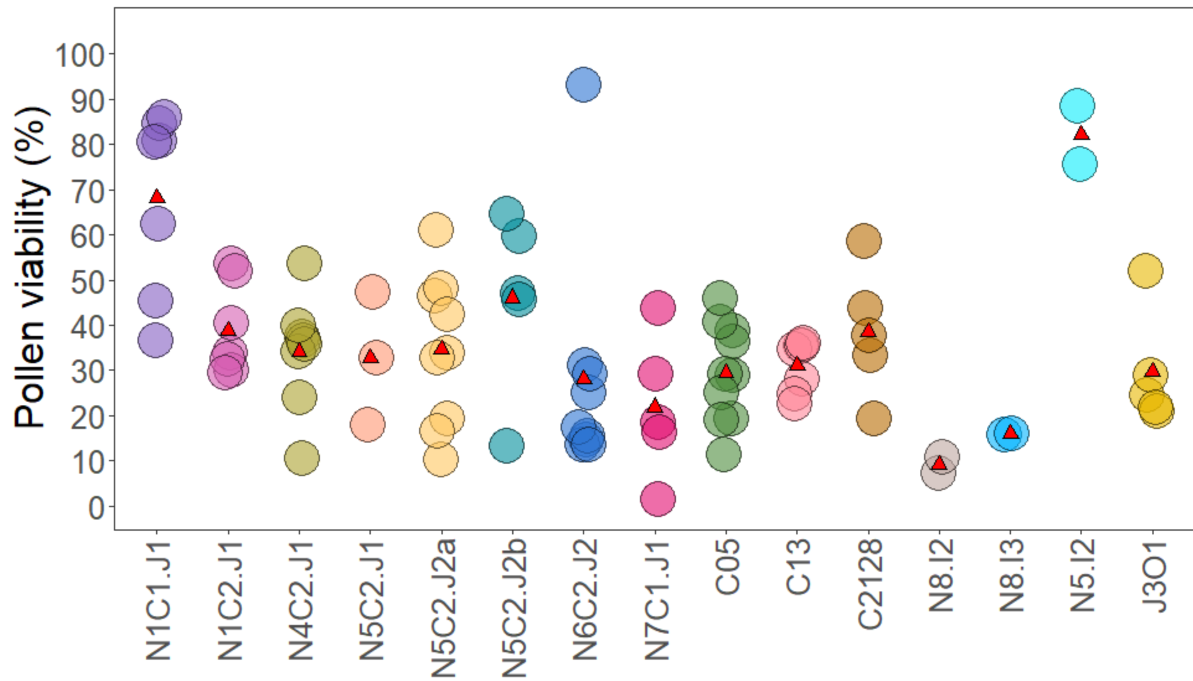
Supplementary Figure 1. Allohexaploid lines ranked from low to high total number of single-copy events affecting chromosome counts. *Brassica* allohexaploid genotypes NCJ: N1C1.J1, N1C2.J1, N5C2.J2a, N5C2.J2b; Carirapa: C05, C13, C2128, and junleracea: J3O1. Each line per genotype is shown as "_" followed by the number of the line (1 to 5). Each line represents the total number of events in all the individuals analyzed (located in the centromere region, extra copy or missing copy).



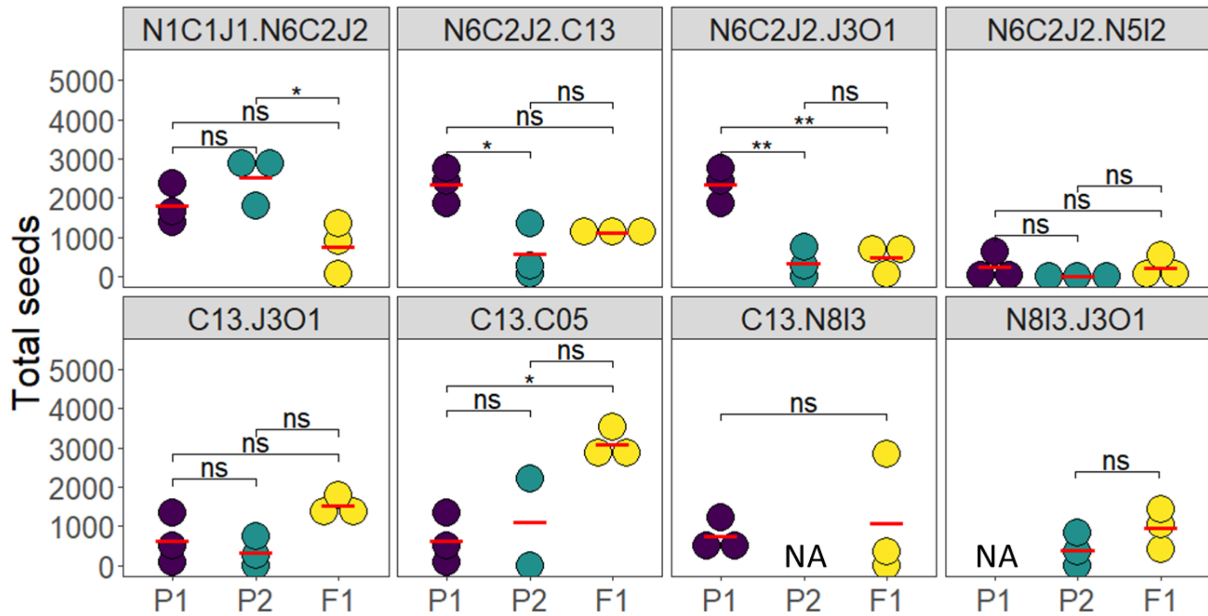
Supplementary Figure 2: Frequency of copy number variation (CNV) event size per allohexaploid type. Allohexaploid type is shown according to the legend: NCJ in pink, carirapa in purple, and junleracea in dark green. The size is represented in megabases (Mb) distributed in 1 Mb bin width for each of the CNV type: deletion, duplication, missing copy, and extra copy.



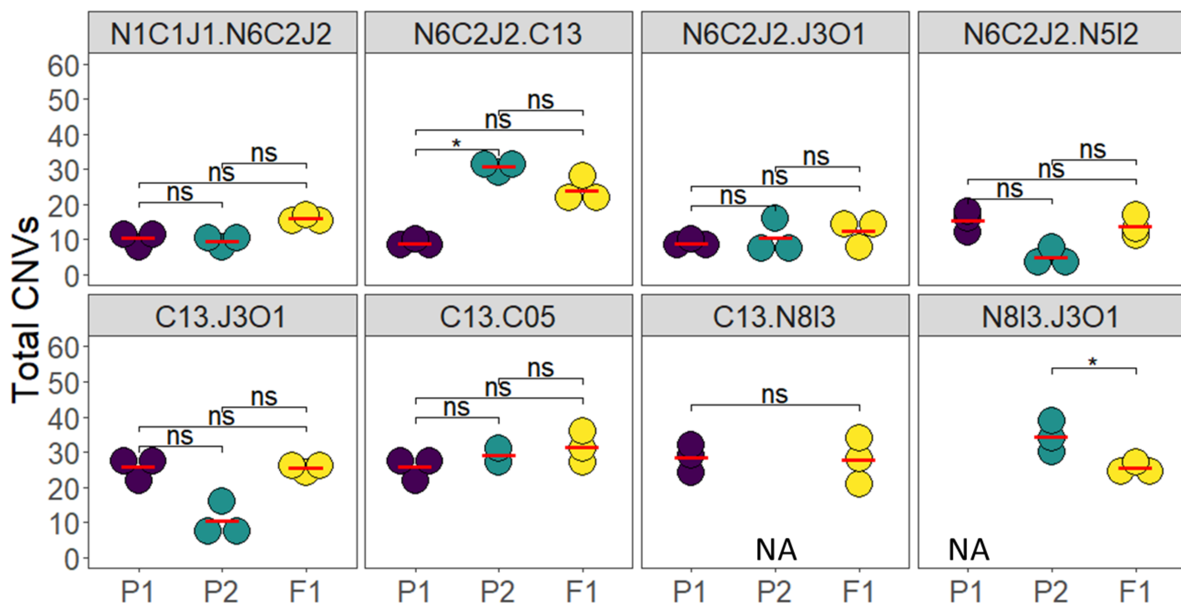
Supplementary Figure 3. Size comparison between allohexaploid types for different copy number variation (CNV) events. Each dot represents a single event scored per allohexaploid type. Allohexaploid type is colored according to the legend: NCJ in pink, carirapa in purple, and junleracea in dark green. Mean values per group is shown by a red horizontal line. Comparisons were made within the groups and between the allohexaploid type (Kruskal-Wallis test: deletion p = 1.3e-19, duplication p = 1.4e-6, missing copy p = 1.3e-6, extra copy p = 4.7e-3). Multiple pairwise comparisons were done using Dunn´s test with the level of significance shown in brackets as: “ns” (not significant) = p > 0.05, “*” = p ≤ 0.05, “**” = p ≤ 0.01, “****” = p ≤ 0.0001.



Supplementary Figure 4. Percentage of pollen viability in the different allohexaploid genotypes used for crossing. Each dot represents the percentage of pollen viability per individual from the *Brassica* allohexaploid genotypes NCJ: N1C1.J1, N1C2.J1, N5C2.J1, N5C2.J2a, N5C2.J2b, N6C2.J2, N7C1.J1; Carirapa: C05, C13, C2128, naponigra: N8.I2, N8.I3, N5.I2; and junleracea: J3O1. Mean per genotype is represented by a red triangle.



Supplementary Figure 5: Total seeds produced by parental genotypes (P1 and P2) and their corresponding F¬1 hybrid in Brassica allohexaploids. The genotype combination from each of the F1 hybrids is represented at the top of each graph. P1 and P2 correspond to the genotype used in the combination and are the first and second genotype named in the cross, respectively. Each dot represents one plant per group. Statistical differences (Student's pairwise t-test, unpaired t-test) between parents and hybrids are represented by brackets. Ns = Not significant. NA = Not analyzed. Average value per group is represented by a red horizontal line. NA = genotype not analyzed.



Supplementary Figure 6: Total copy number variants (CNVs) in allohexaploid parents compared to their F_1 hybrids. Each dot represents one plant per group. Statistical differences between parents and hybrids are represented by brackets (Dunn´s test), ns = not significant. NA = not analyzed. Average value per group is represented by a red horizontal line.

Supplementary Tables 1, 2, 3, 4, and 9 were not included due to the extensive length of them.

Supplementary Table 5: Outcome of the multiple linear regression model analysis to estimate the effects of maternal (♀) and paternal (♂) genotype used in the crossing of different Brassica allohexaploids. Allohexaploid types are the product of crossing different parental species: NCJ = *B. napus* × *B. carinata* × *B. juncea*; carirapa = *B. carinata* × *B. rapa*; naponigra = *B. napus* × *B. nigra*, and junleracea = *B. juncea* × *B. oleracea*. In green are highlighted the genotypes with a significant effect. Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1.

Coefficients:

	Estimate	Effect	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	1.0	1.0	0.324148	2.935	0.00431	**
♀N1C2.J1	-0.3		0.289058	-1.157	0.25044	
♀N4C2.J1	-0.8	0.12	0.254457	-3.254	0.00165	**
♀N5C2.J1	-0.8	0.13	0.250437	-3.288	0.00148	**
♀N5C2.J2	-0.8	0.15	0.239152	-3.36	0.00118	**
♀N5C2.J2_2	-0.1		0.333906	-0.423	0.67348	
♀N6C2.J2	0.6		0.398732	1.446	0.15195	
♀N7C1.J1	-0.9	0.01	0.302932	-3.124	0.00246	**
♀C05	-0.7	0.28	0.252007	-2.664	0.00928	**
♀C13	-0.3		0.299777	-0.929	0.3558	
♀C2128	-0.7	0.25	0.258347	-2.701	0.00837	**
♀N8.I1	-0.9	0.04	0.266088	-3.428	0.00095	***
♀N8.I2	-0.5		0.3113	-1.745	0.08476	.
♀N8.I3	-0.1		0.315744	-0.345	0.73122	
♀N9.I2	-1.3	-0.32	0.265272	-4.785	7.35E-06	***
♀J3O1	-0.6		0.415855	-1.529	0.12997	
♂N1C2.J1	0.6		0.307765	1.976	0.05144	.
♂N4C2.J1	0.2		0.33554	0.545	0.58736	
♂N5C2.J1	0.9	1.82	0.330742	2.614	0.01062	*
♂N5C2.J2a	0.6		0.396066	1.451	0.15047	
♂N5C2.J2b	1.0	1.95	0.440797	2.273	0.02562	*
♂N6C2.J2	0.3		0.315936	0.901	0.37036	
♂N7C1.J1	0.0		0.271563	-0.007	0.9947	
♂C05	0.2		0.254003	0.851	0.39716	
♂C13	0.3		0.274487	1.239	0.21882	
♂C2128	0.5		0.286036	1.597	0.11407	
♂N8.I2	-0.3		0.282734	-0.998	0.32123	
♂N8.I3	-0.1		0.32234	-0.186	0.8532	
♂N5.I2	-0.1		0.252044	-0.59	0.55681	
♂J3O1	0.3		0.279578	0.903	0.3693	

Supplementary Table 6: Outcome of the multiple linear regression model analysis to estimate the effect of *Brassica* parents in common in a cross, together with the maternal (♀) and paternal (♂) genotype effect within NCJ allohexaploid genotypes. NCJ allohexaploids were produced by the cross between *B. napus* × *B. carinata* × *B. juncea.* In green are highlighted the genotypes with a significant effect. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

Coefficients:

	Estimate	Effect	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	2.0		0.9	2.264	0.047	*
Parent_common1	-0.8	1.2	0.5	-1.501	0.1642	
Parent_common2	-1.1	1.0	0.6	-1.843	0.0952	.
Parent_common3	-3.2	-1.1	1.0	-3.122	0.0108	*
♀N1C2.J1	-0.6	1.4	0.7	-0.87	0.4048	
♀N4C2.J1	-1.1	0.9	0.6	-1.891	0.0879	.
♀N5C2.J1	-1.2	0.8	0.8	-1.56	0.1498	
♀N5C2.J2a	-1.4	0.6	0.5	-2.853	0.0172	*
♀N5C2.J2b	0.0	2.1	0.5	0.09	0.9297	
♀N6C2.J2	-0.3	1.7	0.8	-0.348	0.7354	
♀N7C1.J1	-1.5	0.5	0.7	-2.219	0.0508	.
♂N1C2.J1	0.8	2.8	0.9	0.849	0.4156	
♂N4C2.J1	0.1	2.1	1.2	0.066	0.9484	
♂N5C2.J1	1.1	3.1	1.0	1.128	0.2856	
♂N5C2.J2a	1.0	3.0	0.9	1.094	0.2997	
♂N5C2.J2b	2.7	4.7	0.9	2.966	0.0141	*
♂N6C2.J2	0.6	2.6	1.0	0.648	0.532	
♂N7C1.J1	-0.4	1.6	0.9	-0.484	0.639	

Supplementary Table 7: Outcome of the linear regression model analysis to estimate (a) genotype specific effect, (b) allohexaploid type, (c) *Brassica napus* genotype effect, (d) *Brassica carinata* genotype effect, (e) *Brassica juncea* genotype effect in the total of CNVs in NCJ allohexaploids. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

a) Genotype specific model:

Coefficients:

	Estimate	Effect	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	5.5		0.6732	8.108	4.30E-14	***
N1C2.J1	6.0	11.5	1.0546	5.71	3.85E-08	***
N5C2.J2a	3.9	9.3	1.0132	3.811	0.000182	***
N5C2.J2b	10.3	15.7	1.1392	9.008	< 2e-16	***
N6C2.J2	6.6	12.1	1.0664	6.21	2.82E-09	***
C05	19.4	24.9	1.333	14.57	< 2e-16	***
C13	23.9	29.4	1.4319	16.692	< 2e-16	***
C2128	13.0	18.5	1.2126	10.721	< 2e-16	***
J3O1	15.2	20.7	1.3471	11.277	< 2e-16	***

b) Allohexaploid type model:

Coefficients:

	Estimate	Effect	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	24.3		0.8452	28.763	<2e-16	***
Junleracea	-3.7	20.7	1.7007	-2.153	0.0325	*
NCJ	-13.5	10.9	0.949	-14.179	<2e-16	***

c) *Brassica napus* genotype effect:

Coefficients:

	Estimate	Effect	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	12.5		0.5332	23.481	< 2e-16	***
N1	-3.9894	8.5	0.874	-4.564	1.21E-05	***
N5	-0.44	12.1	0.943	-0.467	0.642	

d) *Brassica carinata* genotype effect:

Coefficients:

	Estimate	Effect	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	5.5		0.7794	7.003	1.48E-10	***
C2	6.6917	12.2	0.8679	7.71	3.78E-12	***

e) *Brassica juncea* genotype effect:

Coefficients:

	Estimate	Effect	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	8.5		0.696	12.256	< 2e-16	***
J2	3.8427	12.4	0.8204	4.684	7.38E-06	***

Supplementary Table 8: Outcome of the linear regression model analysis to estimate the maternal parent effect in the production of viviparous seeds after crossing different *Brassica* allohexaploids. NCJ allohexaploids were produced by the cross between *B. napus* × *B. carinata* × *B. juncea.* Highlighted in yellow are the genotypes with a significant effect. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

Coefficients:

	Estimate	Effect	Std. Error	t value	Pr(>|t|)	Significance
(Intercept)	13.0471	13.0	4.4707	2.918	0.00437	**
Parent1N1C2.J1	7.9467	21.0	7.2434	1.097	0.27531	
Parent1N4C2.J1	16.6329	29.7	6.7802	2.453	0.01595	*
Parent1N5C2.J1	-13.0471	0.0	9.2166	-1.416	0.16009	
Parent1N5C2.J2	18.4336	31.5	6.7802	2.719	0.00776	**
Parent1N5C2.J2_2	-11.1675	1.9	7.2434	-1.542	0.12639	
Parent1N6C2.J2	-6.2323	6.8	7.2434	-0.86	0.39168	
Parent1N7C1.J1	21.0843	34.1	9.2166	2.288	0.02433	*
Parent1C05	7.0508	20.1	8.4826	0.831	0.4079	
Parent1C13	-7.5307	5.5	7.2434	-1.04	0.30108	
Parent1C2128	-10.7961	2.3	7.2434	-1.49	0.13934	
Parent1MSL.Junius	-0.3434	12.7	6.9898	-0.049	0.96091	
Parent1MSL.IX7	-6.7103	6.3	7.5569	-0.888	0.37675	
Parent1MSL.IX13	-8.9387	4.1	7.9557	-1.124	0.26397	
Parent1Ningyou.IX7	-13.0471	0.0	9.2166	-1.416	0.16009	
Parent1J301	-13.0471	0.0	16.7278	-0.78	0.43731	

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