



Challenges and prospects for a potential allohexaploid *Brassica* crop

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Abstract

The production of a new allohexaploid *Brassica* crop ($2n = AABBC$) is increasingly attracting international interest: a new allohexaploid crop could benefit from several major advantages over the existing *Brassica* diploid and allotetraploid species, combining genetic diversity and traits from all six crop species with additional allelic heterosis from the extra genome. Although early attempts to produce allohexaploids showed mixed results, recent technological and conceptual advances have provided promising leads to follow. However, there are still major challenges which exist before this new crop type can be realized: (1) incorporation of sufficient genetic diversity to form a basis for breeding and improvement of this potential crop species; (2) restoration of regular meiosis, as most allohexaploids are genetically unstable after formation; and (3) improvement of agronomic traits to the level of “elite” breeding material in the diploid and allotetraploid crop species. In this review, we outline these major prospects and challenges and propose possible plans to produce a stable, diverse and agronomically viable allohexaploid *Brassica* crop.

What is allohexaploid *Brassica*?

“*Brassica* allohexaploid” could refer to any interspecific hybrid combination in the *Brassica* genus where at least two different subgenomes are present in a total of three copies. However, the combination most commonly referred to as a *Brassica* allohexaploid is $2n = AABBC$. The reason for this is the existence of the renowned “Triangle of U”, a genomic

interrelationship between six of the cultivated *Brassica* crop species discovered by early cytogeneticists (Morinaga 1934; U 1935). These species comprise three diploid species with AA, BB and CC genome complements, plus an additional three allotetraploid species with AABB, AACC and BBCC genome complements. The fact that each combination of two genomes can co-exist in an established allotetraploid species has naturally led researchers to think about the possibility of whether these three genomes can therefore co-exist at the allohexaploid level, i.e. as $2n = AABBC$.

So aside from scientific interest, why might we want to attempt to generate allohexaploid *Brassica*? In general, increase in ploidy level can be an advantageous strategy for plants, resulting in increased speciation and diversification rates (Leitch and Leitch 2008; Soltis et al. 2009; Jiao et al. 2011; Tank et al. 2015). Allopolyploids are well known to have greater ranges and/or competitiveness under differing environmental conditions than their parent diploids (Stebbins 1950; Soltis and Soltis 2000), and this effect is widespread across diverse genera (Marchant et al. 2016). This effect may be attributable to the ability of the allopolyploids to capture genetic diversity from the diploid parents (Dubcovsky and Dvorak 2007), to express relevant allelic variants related to adaptation (Griffiths et al. 2019), and to confer increased plasticity in expression of adaptive and functional traits (Wei et al. 2019). In simple terms, allopolyploids can

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often express genetic factors contributed from each of the different diploid progenitor species in response to particular environmental conditions. Hence, if parent A is able to tolerate hot conditions better, but parent B copes better with waterlogging, the combination of both sets of genetic variants in the allopolyploid often allows it to grow under both heat and waterlogging stresses by expression of relevant genetic variants from the inherited diploid progenitor genomes. In this way, allopolyploids can not only colonize both parental ranges, but also exploit new environmental niches. In the case of *Brassica* allohexaploids, all six species in the Triangle of U contain genetic variants and agronomic traits of interest (reviewed by Katche et al. 2019). Potentially, the combination of genetic variation from all six species could result in a crop with increased adaptation and agronomic potential, as well as increased heterosis from the contribution of alleles from an additional subgenome

(reviewed by Zou et al. 2010; Chen et al. 2011b; Gaebelein and Mason 2018).

There are five possible species combinations that are known to be able to produce allohexaploids with a genome complement of $2n = AABBCC$ (Fig. 1). The most commonly attempted and most generally successful is the cross between *B. rapa* ($2n = AA$) and *B. carinata* ($2n = BBCC$), followed by *B. napus* ($2n = AACC$) by *B. nigra* ($2n = BB$), and *B. juncea* ($2n = AABB$) by *B. oleracea* ($2n = CC$) (reviewed by Gaebelein and Mason 2018). These crosses usually involve a direct cross of the diploid species with the allotetraploid to produce allotriploid hybrids ($2n = ABC$), which are then chromosome-doubled with colchicine to produce $2n = AABBCC$ allohexaploids. In order to simplify reference to these cross combinations, we will subsequently refer to these types as carirapa, naponigra and junleracea crosses, respectively. Alternative

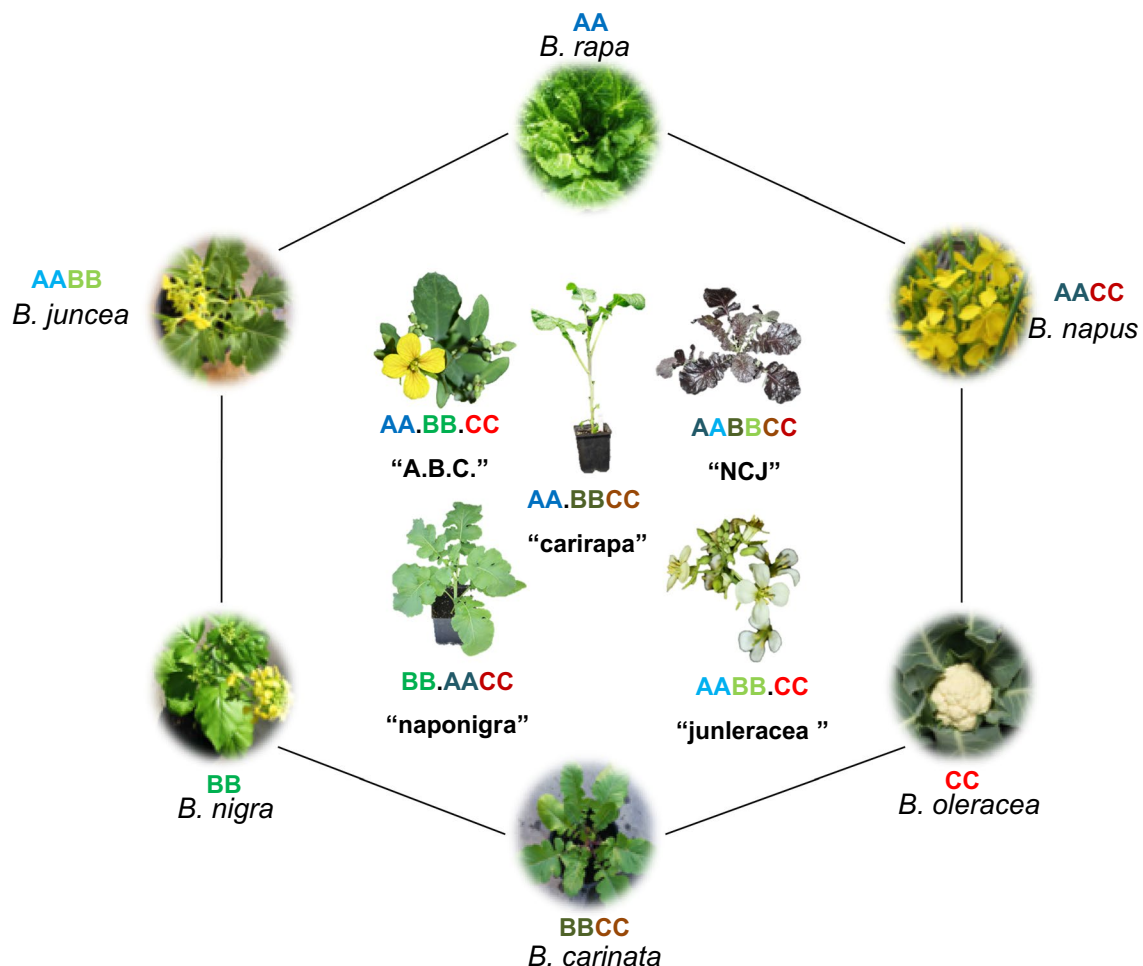


Fig. 1 The traditional "Triangle of U" as a hexagon, with established diploid species *Brassica rapa*, *B. nigra*, *B. oleracea* with AA, BB and CC genome complements, the established allotetraploid species *B. juncea*, *B. napus* and *B. carinata* with AABB, AACC and BBCC genome complements, and possible combinations of allohexaploids

which can be derived from crosses between them: "naponigra" (*B. napus* × *B. nigra*), "junleracea" (*B. juncea* × *B. oleracea*), "carirapa" (*B. carinata* × *B. rapa*), "NCJ" (*B. napus* × *B. carinata* × *B. juncea*) and "A.B.C." (*B. rapa* × *B. oleracea* × *B. nigra*)

methods such as protoplast fusion have also been used to directly unite somatic cells from these species in tissue culture, but to date these methods have not demonstrably resulted in fertile, euploid progeny (reviewed by Gaebelein and Mason 2018). The remaining two methods involve two-step crosses, either between all three diploid species (Zhou et al. 2016) or between all three allotetraploid species (Mason et al. 2012). In the first instance, a synthetic allotetraploid is first produced by crosses between two of the three diploid species, followed by crossing of the synthetic allotetraploid to the third diploid species (e.g. $AA \times CC \rightarrow AACC \times BB \rightarrow ABC \rightarrow AABBCC$). We will subsequently refer to these as A.B.C. hexaploids, following the naming convention of (Zhou et al. 2016). In the second instance, crosses are made in the same fashion between two of the three allotetraploids, following which the resulting hybrid is crossed to the third species (e.g. $AACC \times BBCC \rightarrow CCAB \times AABB \rightarrow AABBCC$). We will subsequently refer to the allohexaploids produced by crosses between the allotetraploid species as NCJ types, referring to the names of the species in the cross combination (*B. napus*, *B. carinata* and *B. juncea*). Crosses between allotetraploids rely on production of unreduced gametes (gametes with the somatic chromosome number, or all chromosomes present in the somatic tissue of the interspecific hybrid; see Bretagnolle and Thompson 1995; De Storme and Geelen 2013; De Storme and Mason 2014 for review) in the cross to restore balanced ploidy level, while crosses between diploids rely on colchicine treatment or other chemical agents to double the chromosome number. Both of these two methods have only ever been successfully carried out using one order of crossing; the one used in the examples above (Mason et al. 2012; Zhou et al. 2016). To date, production of allohexaploids between all species combinations in the Triangle of U has been successfully carried out (reviewed by Chen et al. 2011b; Gaebelein and Mason 2018).

This review paper will outline the major challenges and prospects for production of a novel allohexaploid *Brassica* crop, and summarise progress to date in establishing this new crop species. Potentially, a new allohexaploid crop could combine genetic and trait variation from all six *Brassica* “U’s Triangle” species with added hybrid vigour from the addition of an extra set of alleles. However, we propose that there are three main challenges facing establishment of an allohexaploid crop. Firstly, the provision of sufficient genetic diversity to form the basis for traditional and modern plant breeding and selection approaches; secondly, the restoration of meiotic stability and fertility; and thirdly, the establishment of agronomic traits and yields competitive with established *Brassica* crop types.

The importance of genetic diversity in establishment of a new allohexaploid crop

Genetic diversity is key to species adaptability and fitness, particularly under changing environmental conditions, and a necessary substrate for plant breeding and crop improvement, providing the genetic basis for artificial selection of traits beneficial to humans (Tanksley and McCouch 1997; Tester and Langridge 2010). Introducing genetic diversity from wider germplasm pools, wild and related species into elite crop types is becoming increasingly recognized as critical for modern crop breeding (Xiao et al. 1996; Longin and Reif 2014). Allohexaploids derived from interspecific hybridization may contain beneficial alleles and genetic diversity present in each of the *Brassica* “U’s Triangle” species. *Brassica* species readily hybridize (FitzJohn et al. 2007; Kathe et al. 2019) and resulting resynthesized species can benefit from genomic plasticity obtained from frequent homoeologous sequence exchanges between subgenomes (Schiessl et al. 2018). The ample subgenomic/genomic variations within or between *Brassica* “U’s Triangle” species due to speciation, domestication and geographic differentiation offer a robust potential for crop improvement through introgression of subgenomes, which is very useful in theoretical and applied research (Zou et al. 2010; Liu et al. 2016; Yang et al. 2016a, b). For example, development of a new-type *B. napus* population via introgressions from *B. rapa* and *B. carinata* presented novel allelic combinations, reconstructed linkage disequilibrium patterns and resulted in frequent deletions and duplications, particularly in the C subgenome (Zou et al. 2018).

With respect to *Brassica* allohexaploids, many different approaches can be undertaken to synthesize these new crop types from existing diploid and allotetraploid species (reviewed by Chen et al. 2011b; Gaebelein and Mason 2018). Pyramiding of genetic diversity from the different *Brassica* species can produce allohexaploids with superior genetic variation compared to the existing diploid and tetraploid species. Increased variation can arise from (1) novel interactions between homoeologues derived from the combination of the A, B and C subgenomes in one species (AABBCC) compared to their diploid (AA, BB, CC) or tetraploid (BBCC, AACC, AABB) parental species, (2) the production of diverse hexaploids with subgenomes originating from different progenitor species, such as $A^rA^rB^cB^cC^cC^c$ (A^r genome from *B. rapa*, and B^c/C^c genomes from *B. carinata*), $A^nA^nB^nB^nC^nC^n$ (B^n genome from *B. nigra*, and A^n/C^n genomes from *B. napus*), and $A^jA^jB^jB^jC^oC^o$ (C^o genome from *B. oleracea*, and A^j/B^j genomes from *B. juncea*), (3) incorporation of variation from different genotypes of the parental species,

(4) novel combinations produced by intercrossing and recombination between allohexaploid types (e.g. between $A^nA^jA^nA^jC^nC^o$ and $A^rA^nB^cB^nC^cC^n$), and (5) novel genetic variation induced by “genome shock” following interspecific hybridization and polyploidization (Xiong et al. 2011; Zou et al. 2011, 2018; Song and Chen 2015; Ding and Chen 2018).

Novel traits and hybrid vigour, as results of genetic diversity

With increased genetic diversity, novel trait variation and strong hybrid vigour can be generated in a new allohexaploid *Brassica* species. Firstly, specific favourable traits present in the parental species can be introduced to the derived hexaploids. These may include traits such as heat and drought resistance from *B. juncea* (Schelfhout et al. 2006; Paritosh et al. 2014), pod shattering and drought resistance from *B. carinata* (Jiang et al. 2007; Dhaliwal et al. 2017; Raman et al. 2017), resistance to *Sclerotinia* from wild *B. oleracea* (Ding et al. 2013), and resistance to blackleg from *B. nigra* (Zhu et al. 1993; Gaebelein et al. 2019a). Secondly, superior traits in the hexaploids in comparison to the parental species may emerge, such as increased biomass and “fixed heterosis” (Ramsey and Schemske 2002; Abel et al. 2005; Comai 2005). Thirdly, novel traits such as specific quality components, plant architecture, male sterility, and some pest or disease resistances may appear in the hexaploids due to induced reorganization of genome components (e.g. chromosome rearrangements), a phenomenon which has frequently been observed in synthetic *B. napus* (Schranz and Osborn 2000; Zou et al. 2011; Chatterjee et al. 2016; Pires and Conant 2016; Jiang et al. 2018). Lastly, hybrid vigour may be present between hexaploids with different origins because of “intersubgenomic heterosis” (Zou et al. 2010), although heterosis between different types of hexaploids has not yet been reported. Fully exploiting the value of this genetic diversity with respect to traits and hybrid vigour is a valuable long-term goal. In the short term, the major “genome shock” resulting from distant hybridization may severely affect the genome stability of the new allohexaploid species and its reproductive performance (Ramsey and Schemske 2002; Yao et al. 2012; Mwathi et al. 2017; Gaebelein et al. 2019b). Speed breeding technologies such as genome-wide marker-assisted selection and recurrent population selection, and genome editing with a focus on important genes or genomic regions related to genome stability and important economic traits could in future be undertaken to explore the value of the genetic diversity present in the *Brassica* hexaploids (Ahmar et al. 2020; Yang et al. 2020a). At the same time, selection under specific controlled environments such as salt, heat, drought and heavy disease stress conditions

would be useful to identify resistances, accelerate domestication and promote “de novo domestication” and plasticity (Hickey et al. 2019).

Producing allohexaploids: interspecific hybridization and chromosome doubling

Although interspecific hybridization in *Brassica* is relatively easy and achievable (FitzJohn et al. 2007), there are still major challenges facing the production of a genetically diverse pool of *Brassica* hexaploids. These include species- and genotype-specific hybridization barriers, low efficiency of chromosome doubling, rapid chromosomal elimination from genomes of the parental species, and the possible occurrence of de novo self-incompatibilities and cross-incompatibilities between different germplasm pools (Song et al. 1995; Udagawa et al. 2010). To date, as a result of these barriers, the number of available hexaploid genotypes from different origins is still limited. However, there is also great potential to further broaden the genetic diversity of the *Brassica* hexaploid germplasm pool by improving interspecific cross-compatibility and chromosome doubling efficiency.

Understanding the main factors influencing interspecific cross-compatibility is essential for developing allohexaploids, as hybrids were only successfully produced in some combinations or in an inherent direction within a study (FitzJohn et al. 2007). Firstly, the cross-compatibilities between the *Brassica* species are known to vary (Gupta et al. 2016; Wang et al. 2016). For instance, embryo rescue is generally necessary for crosses between *B. oleracea* and other *Brassica* species, or between *B. nigra* and other *Brassica* species, among others (Zhang et al. 2004; Sharma et al. 2017). By contrast, embryo rescue is not necessary for most crosses between *B. carinata* and *B. rapa* (Jiang et al. 2007), *B. juncea* and *B. rapa* (Teng et al. 2018), and *B. napus* and *B. rapa* (Bing et al. 1996; Hansen et al. 2001). Understanding the genetics and molecular mechanisms underlying interspecific incompatibility and compatibility would help to increase the number of possible cross combinations and hence overall genetic diversity (Bernacchi and Tanksley 1997). Secondly, cross-compatibility also varies between different genotypes within a species (Jiang et al. 2007; Udagawa et al. 2010). For example, although most *B. rapa* cultivars are incompatible with *B. oleracea*, an Indian “yellow sarson” oilseed cultivar seemed to mostly be compatible with *B. oleracea* (Udagawa et al. 2010). Therefore, selection of genotypes with relatively high cross-compatibility as bridges could improve the crossability between species. Thirdly, cross-compatibility also depends on cross direction (Chen et al. 2011b). For example, in several previous attempts of interspecific hybridization between *B. napus* and other similar *Brassica* species, it was

often more successful when *B. napus* used as the female parent than vice versa (Scheffler and Dale 1994; Schelfhout et al. 2006; Pradhan et al. 2010). Niemann et al. (2014) made a similar observation on the cross-compatibility of reciprocal crosses between self-compatible *B. napus* var. *oleifera* and self-incompatibility *B. rapa* ssp. *pekinensis*. Hybridization between these two species was successful only when the *B. napus* cultivar was used as a maternal parent. The phenomenon is referred to as unilateral incompatibility (Abdalla and Hermesen 1972), which usually occurs in reciprocal crosses between closely related self-compatibility and self-incompatibility parents. The stigma of the self-incompatibility partner rejects pollen from the self-compatibility partner, whereas the converse is feasible (Kemp and Doughty 2003). Also, it has been reported that the difference in gene expression between a carirapa (*B. carinata* × *B. rapa*) allohexaploid and its male parent was more significant than that with its female parent (Zhao et al. 2013), which may be partly attributable to maternal effects. However, since data for other cross combinations are limited, further studies are required to confirm that epigenetic and gene expression changes in the possible species combinations are nonrandom. In conclusion, studies on cross-compatibility in *Brassica* species not only help to select compatible parental components and the determination of correct cross direction, but also provide guidance on combining characters from self-compatibility and self-incompatibility parents.

Additionally, the morphotypes used may have an indirect impact on interspecific hybridization. For instance, crosses between *B. napus* and *B. oleracea*, especially with *B. oleracea* var. *capitata* (cabbage), are generally difficult to obtain, even if *B. napus* is used as the maternal parent (Scheffler and Dale 1994; FitzJohn et al. 2007). U (1935) produced hybrids from the combinations of *B. napus* var. *oleifera* (rapeseed) with *B. oleracea* var. *gemmifera* (Brussels sprouts) and *B. oleracea* var. *acephala* (kale), but failed to obtain any hybrids when cabbage was used as either the female or the male parent. However, according to Chiang et al. (1977), interspecific hybridization between *B. napus* and *B. oleracea* var. *capitata* succeeded when "Badger Shipper", a cabbage cultivar derived from the natural hybrid of cabbage and Jersey kale, was used as the pollen parent. This cabbage cultivar seemed to have genetic diversity necessary for interspecific compatibility (Wall and York 1960), indicating the potential to make the combinations successful by using particular intraspecific cultivars. Besides, the efficiency of chromosome doubling after interspecific crosses varies (Geng et al. 2013), which may be also due to genotype-specific effects. It is common to use the chemical colchicine for chromosome doubling, but this chemical generally shows a low rate of successful chromosome doubling, and can additionally cause damage such as chromosome rearrangements and other mutations in treated plants (Zhang et al. 2003; Gu

et al. 2004; Xu et al. 2007). Mitosis-inhibiting herbicides such as dinitroanilines are considered to be standard alternatives for colchicine (reviewed by Dhooghe et al. 2011), which could be also used in *Brassica*. Although spontaneous chromosome doubling of interspecific hybrids is very rare in *Brassica*, spontaneous chromosome doubling has also been reported in an F₁ hybrid (ABBC) between *B. carinata* and *B. juncea* to yield an octaploid plant (AABBBBCC) (Chatterjee et al. 2016). Possibly, selection of hybrid and parent types with the ability to either undergo spontaneous chromosome doubling or to produce high frequencies of unreduced gametes as cross bridges would dramatically increase the efficiency of chromosome doubling to resynthesize hexaploids. In summary, although *Brassica* hexaploids have great potential for rich genomic variation, more research and investment in this future crop, including intensive crossing and population-based selection with the assistance of modern technologies are needed to achieve a diverse hexaploid population with rich genetic and favourable phenotypic variation at the species level.

The challenge of meiotic stability in allohexaploids

Meiotic stability is thought to be the single greatest challenge facing the establishment of polyploids in nature (Pelé et al. 2018). After allopolyploid formation, two or more genomes including a full complement of chromosomes are united together in the same cell. There are many regions containing similar sequences among chromosomes originating from the alternative parental genomes. During meiosis, these "homoeologous" chromosome regions are often not correctly identified and hence multiple or illegitimate chiasmatic associations are formed in prophase I (Fig. 2). This can result in chromosome mis-segregation, and subsequently generation of genetically unbalanced gametes with loss and duplication of chromosomes or chromosome regions. This results in loss or reduction of fertility and reduced fitness in newly resynthesized allopolyploids and may be linked to failure to establish successful populations in natural environments (Pelé et al. 2018). Therefore, it is not surprising that this is also a major challenge to the production of artificial allopolyploid crops, such as synthetic *Brassica* allohexaploids. Similar difficulties were also encountered by researchers in the early days of triticale, a human-made hybrid between wheat and rye (O'Mara 1953). Fortunately for triticale, breeding even in the days before the molecular genetics and genomics revolution was able to successfully restore fertility and meiotic stability in this crop, albeit over a long period of time (Gupta and Priyadarshan 1982; Oetler 2005). There is also some evidence that the genome stabilisation process can occur rapidly, either immediately

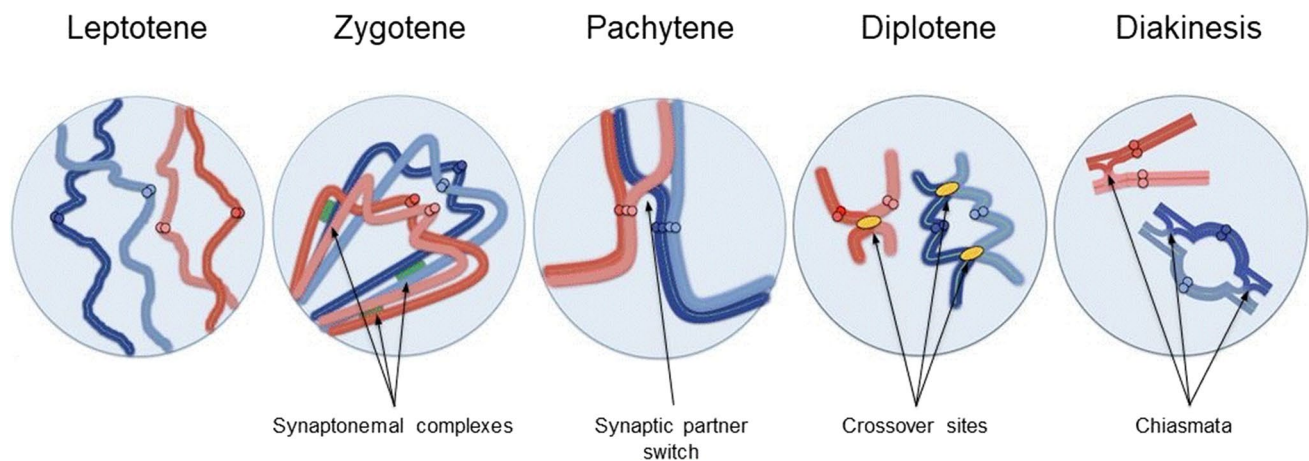


Fig. 2 A model of chromosomal behaviour during prophase I of meiosis in allopolyploids. During premeiosis interphase, sister chromatids are generated and associated by cohesins. This indicates the onset of leptotene, during which genetic recombination is initiated by the formation of double-strand break (DSB). Subsequently, the telomeres form a classical bouquet, and the homologous and homoeologous chromosomes start to synapse via the formation of the synaptonemal

complex (SC) in zygotene. The SCs between homoeologous chromosomes in allopolyploids leads to synaptic partner switches at pachytene. During diplotene, the SC breaks down but homologues remain associated at crossover (CO) sites. In diakinesis, the homologous chromosome pairs begin to separate, except at sites where COs have occurred, resulting in establishment of physical connections known as chiasmata

(Gupta et al. 2016) or within a few generations, putatively through selection for allelic variants of meiosis genes (Lloyd and Bomblies 2016). Of course, natural, presumably meiotically stable polyploids are also highly abundant (Jiao et al. 2011; Barker et al. 2016), suggesting that while meiotic stability may be a barrier to species establishment, it is one which is quite often overcome in natural (and agricultural) environments.

To date, resynthesized and synthetic *Brassica* allopolyploids have been observed to be extremely unstable (Song et al. 1995; Szadkowski et al. 2011), while naturally occurring allotetraploids *B. juncea*, *B. carinata* and *B. napus* are fully stable and fertile. There are two major hypotheses regarding the differences in stability observed between natural and synthetic polyploids: (1) immediately stable allopolyploids could form due to inheritance of particular genetic variants or genomic structures from the parent species; or (2) some sort of novel mutation may have occurred following allopolyploidization which may play a critical role in meiotic regulation and genome stability in naturally occurring *Brassica* polyploids.

As yet, the mechanisms conferring meiotic stability to de novo *Brassica* allohexaploids are unknown. However, progress is rapidly being made at uncovering the genetic variants and genomic factors responsible for these effects. As the segregation and transmission of chromosomes is a highly controlled process regulated by a series of meiosis-related genes, alterations in the regulation of these genes in synthetic *Brassica* allopolyploids could represent the major factor influencing meiotic stability. And there is evidence that several typical meiosis-related genes are correlated

with meiotic stability in synthetic *Brassica* allopolyploids (Table 1). In the study of Gaebel et al. (2019b), several candidate genes were implicated in the fertility and genome stability of NCJ allohexaploids. Yang et al. (2020b) also found that repressed expression of meiosis-related genes was associated with cytological abnormalities in *Brassica* allotriploids (ABC). In future, it seems likely that it will be possible to screen parental germplasm for desirable allelic variation, or to utilize known sources of meiotic stability to further expand the germplasm pool and introgress useful characters while maintaining the critical trait of genome stability and fertility in the allohexaploid lines.

The relationship between fertility and meiotic stability

The relationship between fertility and meiotic stability is a critical one: in the absence of meiotic stability, fertility is expected to be low, or even non-existent. Selection for increased fertility may therefore be expected to also select for increased meiotic stability, a common assumption in both evolutionary and breeding experiments (De Storme and Mason 2014). However, whether or not this association always holds true for *Brassica* allopolyploids is debatable. Mason et al. (2014) found little association between chromosome loss, homoeologous recombination frequency and fertility in a segregating F_2 population of NCJ allohexaploids. A recent study also indicated that loss of chromosomes or chromosome segments was not associated with fertility in an allohexaploid *Brassica* doubled haploid (DH) population

Table 1 Putative genes responsible for meiotic stability in *Brassica* allohexaploids, based on publications of Osman et al. (2011), Gaebelein et al. (2019b) and Yang et al. (2020b)

| Gene | Protein Function ^a | A genome homoeologues ^b | B genome homoeologues ^b | C genome homoeologues ^b |
|----------------|---|------------------------------------|---|---|
| <i>SPO11-1</i> | Required for DSB formation | A1, BjuA10 | BniB1, BjuB1, BniB8 | BolC1, BnaC1 |
| <i>SPO11-2</i> | Required for DSB formation. SPO11-1 and SPO11-2 have overlapping functions | A9, BjuA1, BjuA3 | BniB2, BniB6, BjuB6, BcaB7 | BcaC4, BolC9, BnaC9 |
| <i>PRD1</i> | Required for DSB formation | A3, BnaA2, BjuA9 | B2, BniB1, BcaB1, BniB3, BjuB3, BcaB5, BniB6, BjuB8, BcaB8 | BcaC1, BolC3, BnaC3 |
| <i>PRD3</i> | Required for DSB formation | A6, A10 | B7, BniB2, BcaB3, BjuB6 | C7, BolC5, BnaC5, BcaC6 |
| <i>MRE11</i> | The Mre11-Rad50-Nbs1/Xrs2 complex directs the processing of DSBs | A10, BjuA1 | B8, BjuB1, BcaB1, BniB2, BjuB2, BniB5, BjuB5, BniB6, BcaB6 | BcaC4, BolC9, BnaC9 |
| <i>RAD51</i> | A homologue of the bacterial RecA protein; catalyzes sister-chromatid and non-crossover (NCO) recombination | A3, A10, BnaA1, BjuA6 | B8, BcaB1, BniB3, BjuB3, BjuB5, BniB6 | BolC3, BnaC3, BcaC4, BolC9, BnaC9 |
| <i>DMC1</i> | A homologue of the bacterial RecA protein; required for inter-homologue recombination | A1, A3, A5, BjuA10 | BniB1, BjuB1, BcaB3, BniB4, BcaB4, BniB5, BjuB5, BcaB6, BniB7, BjuB7, BniB8 | C5, BolC1, BnaC1, BcaC9 |
| <i>MLH1</i> | A homologue of the MutL protein, which is part of the DNA mismatch repair (MMR) system; limits recombination between diverged sequences | A3, BjuA9 | BcaB1, BniB3, BjuB3 | BcaC1, BolC3, BnaC3 |
| <i>MSH2</i> | A homologue of the MutS protein, which is part of the DNA MMR system; inhibits recombination between divergent direct repeats or between homologues from different ecotypes | A1, A3 | BniB1, BjuB1, BcaB4, BniB8 | C1, BolC3, BnaC3, BnaC4, BolC6, BnaC6, BcaC8, BcaC9 |
| <i>MSH4</i> | A homologue of the MutS protein; required for the promotion of CO formation | A8 | BniB1, BjuB1, BcaB3, BcaB4, BniB7, BjuB7, BniB8 | BcaC7, BolC8, BnaC8 |
| <i>ASY1</i> | Required for morphogenesis of the SC | A7 | BniB1, BjuB1, BniB2, BjuB2, BjuB3, BcaB4, BcaB5, BniB7, BjuB7, BniB8 | BnaC1, BnaC2, BcaC2, BcaC3, BolC4, BolC5, BolC6, BnaC6, BcaC8 |

^aProtein functions are proposed on the basis of studies of corresponding proteins and their homologues in other species, especially *Arabidopsis thaliana*^bA genome homoeologues include homoeologues in the A genomes of *B. rapa* (BraA), *B. napus* (BnaA) and *B. juncea* (BjuA); B genome homoeologues include homoeologues in the B genomes of *B. nigra* (BniB), *B. juncea* (BjuB) and *B. carinata* (BcaB); C genome homoeologues include homoeologues in the C genomes of *B. oleracea* (BolC), *B. napus* (BnaC) and *B. carinata* (BcaC). The homoeologues in the A, B and C subgenomes of *Brassica* allohexaploids are based on the results of the “blastn” program (<https://blast.ncbi.nlm.nih.gov>) between corresponding gene sequences in *A. thaliana* and the *B. rapa* reference genome CAAS_Brap_v3.01 (Wang et al. 2011; Zhang et al. 2018), the *B. oleracea* reference genome BOL (Chang et al. 2011; Parkin et al. 2014), two assembled *B. nigra* genomes, ASM168289v1 (Yang et al. 2016a, b) and Bnig_sang_1.1 (Paritosh et al. 2020a), a constructed *B. napus* pan-genome, pan-reference.v1.fa (Song et al. 2021), two assembled *B. juncea* genomes, *Brassica.juncea*_var.*tumida*_T84-66_v1 (Yang et al. 2016a, b) and UDSC_Var_1.1 (Paritosh et al. 2020b), and an assembled *B. carinata* genome, ASM1677196v1 (available from NCBI Assembly Database, submitted by North China University of Science and Technology, Hebei, China). Homoeologues were determined under the rules: e-value < 10⁻⁵

(Yang et al. 2018). By contrast, Gaebelein et al. (2019b) suggested that meiotic stability was the main factor affecting fertility across several generations of self-pollinated allohexaploid plants. Third and fourth self-pollination generation segregating populations in this study had high fertility, such that some of plants even exceeded the parental fertility level. Highly fertile plants showed 88–93% bivalent pairing (23–25 bivalents per pollen mother cell), while plants with lower fertility showed 66–67% bivalent pairing (~17 bivalents per pollen mother cell), demonstrating a strong positive correlation between meiosis and seed setting of the studied hybrids. Similarly, Zhou et al. (2016) also suggested that low fertility of allohexaploid *Brassica* hybrids is based on the highly irregular meiosis. Fertility and meiosis were also linked in *B. rapa* by *B. carinata* allohexaploids, where fertile lines showed 100% bivalent formation, while very few or no seed production was recorded in lines with irregular meiosis (Gupta et al. 2016). The hexaploid hybrids of *B. napus* and *B. nigra* also showed highly irregular meiosis, with non-homologous chromosome pairing between the B genome and the A/C genomes was about 15% in pollen mother cell and no seed set (Gaebelein et al. 2019a).

Rare allelic variants may restore meiotic stability in *Brassica* allohexaploids

Although it is clear that the majority of allohexaploid germplasm produced to date is unstable (reviewed by Gaebelein and Mason 2018), there have also been some promising recent findings. Not only has significant variation for meiotic behaviour and fertility been observed between different allohexaploid genotypes from both the same and different species origins (Tian et al. 2010; Zhou et al. 2016; Mwathi et al. 2017; Gaebelein et al. 2019a, b), but one parent genotype conferring near 100% stability (as assessed by bivalent frequency during metaphase I of meiosis) has also been identified (Gupta et al. 2016). In this latter case, a single *B. rapa* parent genotype was found to confer this high-stability phenotype to all allohexaploids produced from it, regardless of the *B. carinata* parent genotype used. This strongly suggests a major locus in the A genome with a genome-wide effect on chromosome pairing across all subgenomes. Such a locus may work either by discriminating between homoeologous chromosomes, as occurs in bread wheat through the operation of the *Ph1* locus (Griffiths et al. 2006; Bhullar et al. 2014) and in allopolyploid *Arabidopsis* species (Lloyd and Bomblies 2016), or by reduction of CO frequency to one CO per homologous chromosome pair, as occurs in autopolyploid *Arabidopsis* species (Lloyd and Bomblies 2016). Unfortunately, this variant may be rare in *B. rapa*: of the 457 carirapa hexaploid combinations produced by Tian et al. (2010), only 35 combinations (from 25 *B. carinata* and 22 *B.*

rapa parents) successfully produced any hexaploid progeny, and only three combinations produced high frequencies of hexaploid progeny.

Little is known about the locus in *B. rapa*, but similar loci, which are responsible for the control of homoeologous chromosome pairing, were discovered in *B. napus*. Pairing regulator in *B. napus* (PrBn), a major quantitative trait locus (QTL) involved in chromosome pairing, was identified in haploids obtained from F₁ hybrids between a high- and a low-pairing *B. napus* in a previous analysis (Jenczewski et al. 2003). This locus was mapped to chromosome C9, and six minor loci with additive effects on chromosomes A1, C1, C3 and C6 were also identified (Liu et al. 2006). Higgins et al. (2021) recently discovered a significant QTL on chromosome A9, *BnaPh1* that accounted for 32–58% of the variation in the ability to control aberrant pairing between homoeologous chromosomes in a segregating *B. napus* DH population. These studies took the advantage of differences in recombination rates in resynthesized segregating populations, providing useful references for identifying loci controlling homoeologous pairing and evaluating candidate meiosis-related genes. Further analysis of these QTLs will have a positive effect on understanding the mechanism and regulation of meiotic recombination, which may help in finding a solution to the meiotic stability problem faced by allopolyploidy.

Quantitative variation in meiotic behaviour in *Brassica* allohexaploids

Quantitative variation in meiotic behaviour and fertility has also been observed between different genotypes of (*B. napus* × *B. carinata*) × *B. juncea* hybrids (Mwathi et al. 2017), and between microspore-derived progeny sets of NCJ plants from the same genotype (Mwathi et al. 2019). In both cases, it was not clear if chromosome rearrangements, inheritance of particular allelic variants from the parents or both were responsible for differences in meiotic behaviour and fertility. However, a larger and more comprehensive study on segregating mapping populations clarified that both allelic variants of meiosis genes inherited from the allotetraploid parents as well as chromosome rearrangement events and the presence of univalent chromosomes affect meiotic stability and fertility in NCJ hybrids (Gaebelein et al. 2019b). A recent study of naponigra (*B. napus* × *B. nigra*) allohexaploids also identified minor differences between genotypes in meiotic chromosome pairing behaviour (Gaebelein et al. 2019a). Interestingly, these differences appeared to be attributable to the diploid *B. nigra* parent, rather than to *B. napus*, despite the fact that *B. napus* has previously been shown to contain genotype-specific variation for chromosome pairing behaviour (Jenczewski et al. 2003; Liu et al. 2006).

Differences between species in terms of meiotic stability may also exist. Zhou et al. (2016) identified differences in genomic stability and in transmission of all chromosomes from particular subgenomes in carirapa, A.B.C. and junleracea hybrid types. Mwathi et al. (2020) also found meiosis in one junleracea genotype to be much more regular than is generally observed in carirapa or naponigra hexaploid types. Moreover, they suggest that *B. juncea* × *B. oleracea* allohexaploid cross combinations may not be 100% stable, and it seems likely that one or both of these parent species carry common allelic variants which may be of interest for increasing meiotic stability in allohexaploid germplasm pools. To date, very few meiotic observations have been gathered from different genotypes of A.B.C., junleracea, or naponigra hexaploids, so it is difficult to know whether observational differences involving these allohexaploid types are attributable to species-level differences or to genotypes within species.

Some evidence suggests improvement in meiotic stability over generations, even in homozygous material

Although rigorous, high-quality evidence for this effect is still lacking, there is also some suggestion from the literature that it is possible for homozygous allohexaploid lines to stabilize with generational selection. Although the mechanism for this effect is unknown (but would have to be either epigenetic-related to chromosome conformation, or attributable to de novo mutations such as chromosome rearrangement events), several authors have found increasing stability over generations. Tian et al. (2010) found increasing frequencies of hexaploid progeny after generational selection, and similar results have also been observed upon resynthesis of *B. napus*, *B. carinata* and *B. juncea* from hybridization between their diploid progenitor species (reviewed by Prakash et al. 1999).

The main challenge to establishing whether these effects are real or not is (a) clear evidence that pollen contamination between lines is not the cause, thus suggesting introduction of foreign allelic variation as the mechanism; and (b) provision and validation of a convincing epigenetic or chromosome-level mechanism for this effect. In a more recent study, the interspecific hybrids of *B. juncea* × *B. carinata* ($2n = \text{BBAC}$) showed a significant improvement in meiotic pairing behaviour and hybrid fertility over six generations of self-pollination under selection for fertility (Katche et al. 2021). Single-nucleotide polymorphism (SNP) genotyping was performed in these self-pollinated progeny generations to evaluate the presence and absence of A, B and C genomes. Based on the genotyping findings, predicted recombined karyotypes with a total of 20 A-C chromosomes

were proposed to explain the restoration of fertility and meiotic stability over generations. At the chromosome level, the C genome was preferentially lost compared with the A genome in later generations of BBAC, a phenomenon known as biased fractionation, which has also been observed in both synthetic and natural *B. napus* (Samans et al. 2017). Post-polyploidization genome size reduction continued, which may be accompanied by unbalanced A–C translocations in one direction (Gaebelein et al. 2019b), until the rearranged A/C chromosomes achieved a stable status in the sixth self-pollination generation. Allopolyploids typically evolve between species that are genetically similar at least with the same tribe or either within the same genus because cross fertilization barriers increase with increasing genetic distance between species. This suggests that the two genomes that have come together in the modern allopolyploid share certain regions of sequence similarities. Therefore one of the most difficulties in establishing allopolyploidy is stabilising meiosis. However, the reduction of the numbers of functional gene copies for meiotic genes may be an important evolutionary adaptation for meiotic stability in polyploids (Higgins et al. 2021).

Phenotypic and agronomic variation

As a key mechanism of plant evolution and speciation, understanding how polyploidy modifies phenotypic/morphological traits is a major area of concern in plant breeding and evolution studies. The merger of several genomes in a polyploid may affect chromosomal rearrangements, flowering timing, transpiration, photosynthesis, growth rate and reproductive physiology (Balao et al. 2011; Kim et al. 2012; McCarthy et al. 2019). However, less evident effects can also occur. It is believed that polyploidy is a method of increasing plant production potential: genome multiplication can increase possibilities for interaction and expression of gene copies and hence potential heterosis (Washburn and Birchler 2014). The vigorous growth of polyploids is considered favourable, particularly when the plant organs and/or biomass constitute an economically valuable product (Dhawan and Lavania 1996; Majdi et al. 2010). At genetic and genomic levels, polyploidy is well studied. However, few investigations have explored the morpho-physiological and anatomical consequences of polyploidy that could provide a comprehensive understanding of these aspects. In such regard, the *Brassica* crop species represent an excellent model for understanding and exploring polyploidy-induced variation in anatomy, physiology and morphology (Baker et al. 2017).

Although phenotypic data on allohexaploid *Brassica* are limited, some promising findings have been obtained from related studies. Kumari et al. (2018) developed and

characterised hybrids between *B. juncea* and *Sinapis alba* produced via protoplast fusion and found that hybrids were very vigorous and taller than their diploid parents. The hybrids were intermediate in terms of days to flowering and bore bright yellow flowers that were larger than those of the parents, although seed set of the hybrids was greatly reduced. Zhang et al. (2016) also observed that polyploid plants produced from *B. oleracea* var. *alboglabra* and *B. rapa* var. *purpurea* were morphologically intermediate between their parents. They observed remarkable differences in size and shape of the leaves, the size and colour of flowers and stalks, the structure of the inflorescences and the presence of surface wax. Likewise, Li et al. (2018) found that interspecific hybrids between *B. napus* and *B. oleracea* showed lighter green colour and intermediate morphology between their parents, although the newly constructed hybrids had a lower pollen fertility rate compared with their parents. The author also noted that the morphological appearances of these hybrids were especially different from *B. napus*, and as such could serve as a bridge to transfer desirable genes from *B. oleracea* into *B. napus*. In the study of Wei et al. (2016), a new-type *B. juncea* ($A^{r/j}A^{r/j}B^{c/j}B^{c/j}$) obtained through interspecific hybridization, presented abundant genetic diversity as well as good fertility after intensive selection with large populations. With five rounds of recurrent selection on a gene pool of new-type *B. napus* ($A^{r/n}A^{r/jn}C^{c/n}C^{c/n}$), elite lines with high yield and heterosis potential, double low seed quality traits, and other favourable traits as oilseed *Brassica* crop were selected, as well as advanced lines with satisfactory glucosinolate components which would be benefit to resistance breeding (Hu et al. 2019, 2021; Qin et al. 2019). These phenotypic variations in synthetic and resynthesised allopolyploids are of great interest in *Brassica* breeding programs, especially in

improving the plant architecture for enhanced yield. However, due to the unstable nature of resynthesized plants and genomic instability during early polyploidization events, this desirable phenotypic variation is not easy to incorporate into breeding programs.

Combination of three genomes found in the cultivated *Brassica* species comprising the “U’s Triangle” results in complex genetic diversity in allohexaploids and subsequent morphological variations (Fig. 3). The resynthesized plants with desirable morphological and agronomic variation could serve as a breeding material and a rich genetic resource for economically important crops like rapeseed. Wei et al. (2016) resynthesized a new-type of *B. juncea* that was found to be genetically stable in the F_6 generation and showed a substantial increase in seed yield of the hybrids relative to parental accessions. Rahman (2002) found that *Brassica* hexaploids derived from reciprocal crosses between almost zero erucic acid *B. rapa* (0.1%) and high erucic acid *B. carinata* (41.3%) had an intermediate level of erucic acid content (33.4%), which shows potential for introgressing favourable alleles for fatty acid composition into the *Brassica* allohexaploid genetic background. Additionally, after five generations of selfing with selection of the *Brassica* allohexaploid DH population (for more detail: Geng et al. 2013), we have selected three promising lines (19-1-1, 19-12-3, 19-23-1) as breeding materials due to their potential stability, good agronomic characters and high fertility rate. Seed quality especially oil content varied among the existing samples, ranging from 31.9 to 46.5%. The erucic acid content (0.7%) and glucosinolate content (18.2 $\mu\text{mol/g}$ meal) were also satisfactorily low in two self-pollinated progenies, respectively, and the maximum content of oleic acid reached 81.7%. These studied materials can provide unique opportunities to investigate the contribution and interaction of A/B/C genomes

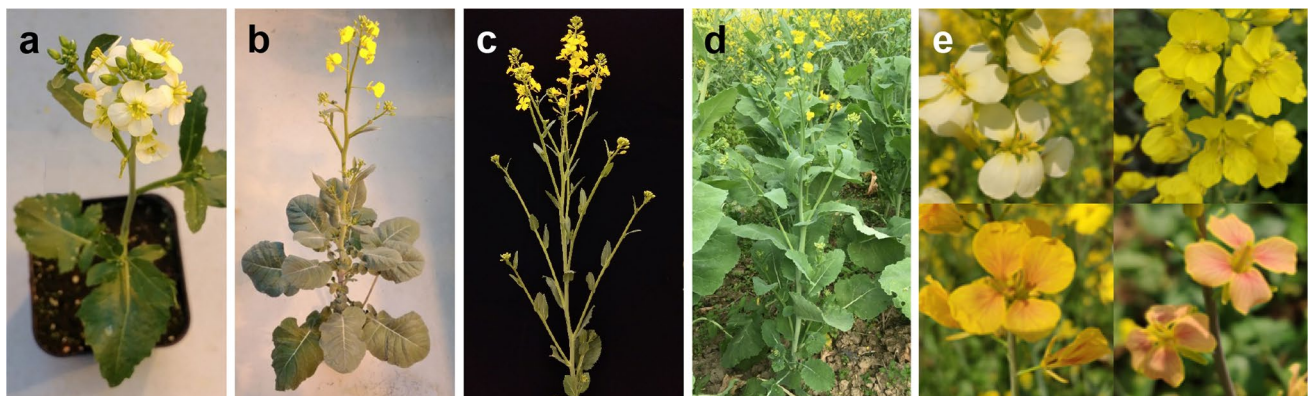


Fig. 3 Diversity of plant morphology and flower colour in *Brassica* allohexaploids and their self-pollinated progenies. **a** Performance of a synthetic junleracea type under greenhouse conditions. **b** Performance of a synthetic carirapa type under greenhouse conditions. **c** Field performance of the self-pollinated progeny of carirapa combi-

nation C28 (described in Tian et al. 2010). **d** Field performance of self-pollinated progenies from a *Brassica* allohexaploid DH population (described in Geng et al. 2013). **e** Flower phenotypes in a recurrent population of *Brassica* allohexaploids (All images belong to the authors' breeding materials)

to seed quality of *Brassica* allohexaploids. This may also provide further genetic improvement such as crossing with hexaploids from parents with low glucosinolates and low erucic acid genotypes, screening with large population segregating, genome editing of targeted genes.

Brassica species have a wide range of accessions, as a result of artificial selection during domestication and cultivation. Six *Brassica* species exhibit economically valuable morphological characteristics such as heading leaves, enlarged organs (e.g. roots, stems and inflorescences) and oilseed (Zhao et al. 2005). Even more interesting is that similar morphotypes can be found in different *Brassica* species with the most common examples being two leafy vegetable types, Chinese cabbage (*B. rapa* ssp. *pekinensis*) and cabbage (*B. oleracea* var. *capitata*). The morphotype diversification in *B. rapa* and *B. oleracea* has been due to the mesohexaploidization of the two *Brassica* genomes combined with convergent subgenome parallel selection of paralogous genes (Cheng et al. 2016). This finding will provide the possibility for using molecular marker assisted selection in breeding of allohexaploid *Brassica* with particular morphotypes. As compared to current allotetraploid and diploid species, allohexaploid *Brassica* can exhibit a greater range of adaptation to new environments, and thus such crops are in demand to counter climate change and to increase per capita food production to counter current global trends (Heinberg and Lerch 2010). In addition, these novel polyploidy *Brassica* lines will provide breeders with access to many thousands of new genes for selection, whereas genetic engineering is currently restricted to one or a few single genes. There is a significant time lag between the development and proof of concept of new transgenes and their incorporation into new transgenic crops (Yang et al. 2010).

Future prospects and challenges

Interspecific hybridization within the genus *Brassica* is potentially valuable and important in *Brassica* breeding due to the potential for hybrid heterosis and extra allelic contributions. Polyploidy in *Brassica* is confined to the tetraploid level, as no higher polyploid species of *Brassica* (e.g., AABBCC) exists in nature, although several other crop species, such as hexaploid wheat (*Triticum aestivum*, genome AABBDD) and hexaploid oats (*Avena sativa* L. and *A. byzantina*, genomes AACCCDD), display higher ploidy levels. The combination of different varieties or subgenomes to produce new allopolyploids in *Brassica* has demonstrated the potential for inter-subgenomic heterosis. Chen et al. (2011a) reviewed the production of a new hybrid type hexaploid *Brassica* via all six-cross combination of *Brassica* species, concluding that new allele combinations could result in better crop improvement and resistance to biotic stress.

Although there have been numerous studies indicating inter-relationships and interspecific cross ability between *Brassica* species, the success of interspecific hybridization still cannot be guaranteed due to different interspecific compatibility (Nishiyama et al. 1991; FitzJohn et al. 2007). Therefore, understanding compatibility varies within species in *Brassica* is of significant importance to syntheses of allohexaploid *Brassica*. As reviewed by Gaebelein and Mason (2018), several combinations of allohexaploid *Brassica* ($2n = AABBCC$) have been established to show increased fertility and meiotic stability, with success rates depending on both the method used for allohexaploid production as well as the genotypes involved. As reported by Gaebelein and Mason (2018), there is also some evidence that development of *Brassica* hexaploid through crosses between *B. carinata* and *B. rapa* followed by chromosome doubling can result in improved yield attributes and seed yield per plant with increasing self-pollination generations. Progress towards successful regeneration of a potentially stable species that could be of benefit to agriculture is expected with further research into this area.

In some studies, allohexaploids have been utilized as bridges between species for the synthesis of novel genotypes containing useful traits such as disease resistance (Rahman 2001; Li et al. 2004) and yellow-seededness; however, these lines often retain chromosomal instability and poor seed production (Meng et al. 1998; Li et al. 2004). Although the genetic mechanism remains a mystery, there is some evidence that meiotic stability in allohexaploids can be conferred by naturally occurring allelic variants present in the progenitor germplasm, as well as for generational improvement in meiotic stability and fertility. Gupta et al. (2016) confirmed that one genotype of *B. rapa* in the cross *B. rapa* by *B. carinata* conferred stable meiosis with a number of different *B. carinata* genotypes in derived allohexaploids. Mwathi et al. (2017) indicated that the genotypes of the first generation hybrids and parents in the cross (*B. napus* × *B. carinata*) × *B. juncea* can affect fertility and meiotic chromosome pairing behaviour in F_2 plants. Recently, Gaebelein et al. (2019b) documented that generational fertility and genomic stability in NCJ hybrids are very likely to be controlled by particular allelic variants of meiosis genes. A number of QTLs are known to be involved in controlling the meiotic behaviour in *Brassica* (Liu et al. 2006) rather than a major gene, supporting this interpretation. Higher stability in a polyploid system may also be established due to the modulation of the function of redundant gene copies, or via the retention of extra meiosis gene copies following polyploidization (Lloyd et al. 2014). A recent study also found that knock out of one copy of meiosis gene *MSH4* appeared to inhibit homoeologous COs between the A and C genomes in *B. napus* allohaploids (Gonzalo et al. 2019); similar manipulation

of meiosis gene copy number in *Brassica* allohexaploids may provide a quick way of restoring meiotic stability in agronomically superior lines.

Interspecific hybridization and polyploidization can play a significant role in enhancing variation and shaping trait novelty to expand crop plant biodiversity. Allopolyploid formation plays a key role in plant speciation and evolution (Jiao et al. 2011) and can contribute to remarkable genome plasticity and enhance hybrid vigour with helpful adaptations to the environment relative to progenitor diploids (Leitch and Leitch 2008). However, genetic factors controlling stability are still major challenging aspects in *Brassica* allohexaploids that need to be explored. Gupta et al. (2016) reported the first stable allohexaploid *Brassica* hybrids which produced progeny with a complete chromosomal complement from all three genomes. The identification of these putatively rare alleles that can regulate meiosis in the allohexaploids that should be present in the diploid or tetraploid progenitors should be a major goal for future research. Testing of mapping populations produced between genotypes with varying meiotic stability and fertility produced from diverse germplasm could be helpful to uncover these factors. Yang et al. (2016a, b) reported the first genetic map of an allohexaploid *Brassica* DH population, which provided the framework for future high-density marker maps as well as QTL analysis. Bulk segregant analysis (BSA) and next generation sequencing are also considered to be efficient strategies to identify large effect QTL alleles in large progeny samples. Moreover, progression of allohexaploid populations to later generations will help in the evaluation of this material at the karyotype and genotype level for increased fertility and stability, as well as future use as a source of disease and insect resistance, edible oil profiles, protein-rich meal and as a raw material for biofuel, in possible uses as oilseed, vegetable, fodder and industrial alternatives. Furthermore, by using genome editing techniques like CRISPR/Cas9, genetic variation and breeding goals can be enhanced in a single generation by editing of targeted genes. Genomic resource analysis of *Brassica* species will finally help to understand the complex homoeologous interactions between different genomes within species and allow the selection of superior breeding material with genetic control and stability in new allohexaploids.

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Declarations

Conflict of interest All authors declared that they have no competing interest.

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