Canola Agronomic Research Program (CARP)
FINAL REPORT

Building bridges to success - Accessing Brassica diploid variation for Canola improvement

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Contents

1 Executive Summary

This is a challenging genetics project that is generating new germplasm to open new opportunities for canola breeding. The project has achieved great success despite the difficulties and set backs stemming from AAFC's response to the Covid-19 pandemic where material from this project was lost during building closure. Critical line development continued and the project focused on the generation of additional backcross 4 individuals to advance the development of the bridging lines. The resources were primarily allocated to the C-genome material as these are the most challenging to develop. Progress has seen the development of bridging lines where all of the A-genome aneuploid chromosomes have been removed and this has resulted in the anticipated increase in fertility. These lines developed are the most promising for further use and they will be used for further crosses with the diverse material collected during this project. Molecular analysis using the Brassica Illumina 90K SNP array was performed and provided comprehensive genotyping allowing selection for the domesticated regions and selection against unwanted chromosomes. Although successful, a new genotyping resource will be required in the future as Illumina has discontinued production of this valuable resource.

The inherent barriers to hybridization between diploid and tetraploid brassica material necessarily results in small population sizes. The small population sizes generated during this project has limited the selection capacity. Future progress will see crosses among backcross 4 lines being conducted to combine the Topas alleles into a single line in addition to selcted diverse germplasm. A trend was noted where the frequency of Topas alleles being transmitted in the BC_4 generation was reduced below the expected frequency and this increased the effort required for bridging line development. The research continues and it is anticipated the bridging line will be developed as planned. Once generated the transfer of the domestication (Topas) alleles into *B. oleracea* germplasm will increase future diversity C-genome in canola.

Following the establishment of an efficient *B. oleracea* transformation protocol, the project succeed in generating the development of targeted mutations in candidate gene selected to reduce the effect of the triploid block in the C-genome material. Unfortunatly, the CRISPR alleles generated in the selected genes resulted in significant pleotrophic effects. This research will continue with the selection of additional gene for CrispR mutations without associated detrimental phenotypes.

The project has successfully achieved its major goals solving the problem of domesticating diploid C-genome material. This represented the greatest hurdle and opens opportunities for increasing tetraploid brassica diversity. The development of the A-genome bridging lines continues and represents a fraction of the challenge in the C-genome.

1.1 Strategy to increase variation in *B. napus*

Genetic diversity is a critical resource for successful breeding programs. *Brassica napus* is a young tetraploid species formed less than the 5,000 years ago through an interspecific hybridization event between the diploids *B. rapa* and *B. oleracea*. The resulting change in chromosome number effectively isolates the tetraploid species from its progenitor diploid species preventing gene flow. The level of genetic variation in *Brassica napus* is reduced by selection for domestication traits and further reduced again by selection during breeding programs (Figure 1). Continued efforts to increase the extent of genetic variation in *B. napus* are made, through mutagenesis, interspecific crosses, and the generation of synthetic *B. napus*. These methods all have drawbacks as new mutations are likely not desirable and the barriers associated with interspecific hybridization are challenging reducing access to desirable alleles.

The overarching goal of this project is to introduce new variation into the important crop species canola through the generation synthetic *B. napus*. The novelty used here, is to first transfer key domestication traits from *B. napus* into both *B. rapa* and *B. oleracea* prior to generation new tetraploid material. This is achieved through the development

of bridging lines that possess the A-genome (*B. rapa*) and C-genome (*B. oleracea*) chromosome numbers but posses key domestication alleles from *B.napus*. Once established, the diploid bridging lines can be crossed into diploid wild crop relatives to access diversity from the wide collection of diploid germplasm. This project has established a collection of brassica crop wild relative expanding on present resources. Due to the dearth of variation present in the canola C-genome, the diversity collection has focused on identifying C-genome variation (Table 2) concentrating on *B. oleracea* and other more distantly related species were also included.

The project is ambitious aiming to generate new genetic material that ultimately can be repeated deployed to increase the extent of genetic variation *B. napus* particularly targeting the germplasm with an annual growth habit that is adapted to the Canadian environment.

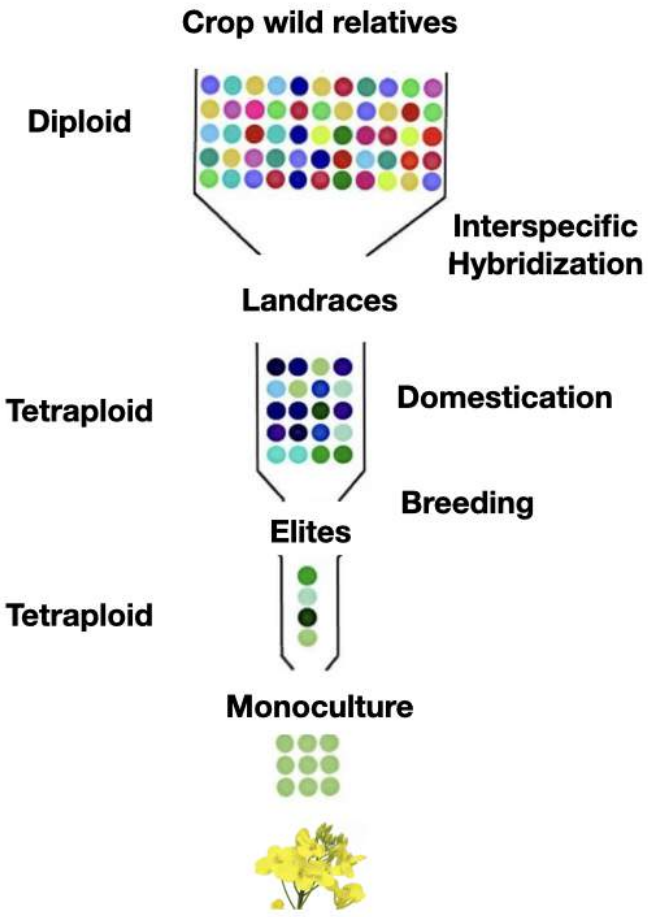

Figure 1: Diagram showing the relative amount of genetic diversity found among Brassica species. The majority of the varaition is found among the older diploid Brassica species and crop wild relatives. The formation of a small number of interspecific crosses amomng these diploid progenitor species formed tetraploid material that instantly become isolated from the diplod material. Selection during the domestication process and further during breeding, has reduced the level of diveristy considerably. It is advantagous to future canola breeding strategies to access the diverity that is currently isolated among its diploid relatives.

2 Introduction

Access to the necessary genetic diversity is key to the success of crop breeding programs and canola possesses low levels of variation (Bus et al., 2011). This is due to the natural history of amphidiploid *Brassica napus* being formed from an interspecific hybridization event between its diploid progenitor species *B. rapa* (A genome) and *B. oleracea* (C genome) (U, 1935) (Figure 2). This hybridization event(s) occurred recently (ca. 2000 years ago) meaning that there has been limited time for mutations and introgressions to occur and natural selection to increase the frequency of alleles we require for further crop improvement. Canola breeders use a range of strategies to overcome this deficiency including mutagenesis, wide genetic crosses and crosses involving wild relatives (Eickermann et al., 2011; Rahman et al., 2015). Crop wild relatives possess greater levels of diversity and the diploid progenitor species which are evolutionary much older than *B. napus*, have had more time to accumulate higher frequencies of valuable alleles (Rahman 2013). These crop relatives act as a rich reservoirs of alleles that offer solutions to breeding objectives if they can be effectively deployed. Unfortunately, significant barriers are present to impede the transfer of useful alleles from crop relatives into *B. napus* (Rahman et al., 2011 & 2015). Major differences separate crop species from their wild relatives (Olsen and Wendel, 2013). These are traits that are often found in many crops species, including polyploidy, flowering time, plant size and shape, shatter resistance and self-compatibility. The factors underlying these differences can be reduced to favourable alleles at only a few loci. They constitute what is called the "domestication syndrome" as these alleles are not competitive under natural conditions and are often referred to as crop domestication traits. These few traits have played an enormous part in the development of agriculture and human civilization (Eshed and Lippman, 2019). The domestication process is often described as a genetic bottleneck since it is associated with a reduction in genetic diversity, a phenomenon that is further exacerbated in modern crops by selection for additional desirable agronomic traits. Unfortunately, these selections can leave modern crops susceptible to disease, insect herbivory and abiotic stresses with no alleles immediately available to resist the stresses. Self-fertilization is often observed in polyploid species as it improves the chances of finding a mate following the genetic isolation caused by the genome duplication event. Additional benefits of polyploidy include the presence of multiple gene copies protecting the polyploid from inbreeding depression that might otherwise become evident following repeated self-fertilization. Domestication traits in *B. napus* include seed size, self-compatibility, adaptations to photoperiod, vernalization, chromosome pairing control, growth vigour, reduced primary dormancy and shatter resistance. Adaptations required to tailor *B. napus* to the Canadian environment are also critical to efficiently evaluate new germplasm. Therefore, elimination of any vernalization requirement and selection for early flowering time while retaining robust growth vigour is highly desirable. This combination of characters forms a baseline requirement that all new *B. napus* material needs to possess to be of interest to Canadian farmers.

However, newly formed polyploids become instantly isolated from their parents in terms of reproduction. This is a major impediment given that whenever useful resistance genes are identified in crop wild relatives they are frustratingly difficult to deploy effectively. This phenomenon is a direct result of post-fertilization barriers that have evolved to promote the success of intraspecific hybridizations which possess balanced chromosome numbers and limit the success of interspecific crosses possessing an unbalance chromosome number. These barriers play a major role in the diversification of species, evolution of genes, and the domestication of crops, but in so doing, limit gene flow from progenitor diploid species into the newly formed polyploidy species. Although the direct introduction of desirable alleles into *B. napus* from its relatives is possible, it is complicated by the union of imbalanced gametes resulting in triploid plants and sterility. Some success has been achieved by generating synthetic *B. napus* lines by directly crossing *B. rapa* with *B. oleracea* and doubling the chromosome number to promote disomic inheritance and genome stability. However, the breeding potential of the resulting synthetic lines are difficult to evaluate as they are often sterile, exhibit self-incompatibility, flower late and require vernalization. In other words, they possess the exact

opposite characteristics for domesticated *B. napus* adapted to the Canadian environment. For this reason, their generation and use in breeding programs is seldom.

Recent genome analyses in Brassica species has elucidated the DNA sequence of *B. rapa*, *B. oleracea* and *B. napus* (Wang et al., 2011, Parkin et al., 2014, Chalhoub et al., 2014). These advances have led to the generation of key genomics infrastructures that allow high-throughput genotyping strategies to support marker-assisted selection including the Brassica 60K SNP Array (Clarke et al., 2016). These genomics resources are now routinely used to characterize defined genetic populations. Detailed results from the molecular characterization of *B. napus* Nested Association Mapping (NAM) populations highlights that the *B. napus* C-genome in particular, as lacking genetic diversity with large chromosomal regions in linkage disequilibrium making this sub-genome an obvious target for improvement. Genetic mapping of key domestication traits in *B. napus* has identified the location of loci controlling vernalization (Teutonico & Osborn, 1995), flowering time (Raman et al., 2013), seedling vigour (Robinson, unpublished), self-incompatibility (Conner et al., 1998) and control over chromosome pairing (Higgins et al., 2020) providing a foundation to understand and manipulate these traits. Recent work at SRDC has integrated this information onto a common marker framework aiding marker-assisted selection (MAS). Additionally, genomic resources can be used to transfer knowledge from model organisms such as *Arabidopsis thaliana* where genetic dissection of traits is more rapid and it is now a relatively simple process to identify Brassica homologues to Arabidopsis alleles using alignment algorithms.

To facilitate access to diploid Brassica alleles from wild species, we have pursued a strategy to exploit the work already done by nature and built upon by plant breeders both ancient and modern. Rather than generate synthetic *B. napus* directly from the diploid progenitor species (*B. rapa* and *B. oleracea*), we have first introduced the desirable domestication alleles from the *B. napus* A-genome and C-genome into *B. rapa* and *B. oleracea* respectively (Figure 1). Ultimately, creating two diploid species that can be used as bridges to access additional A- and C-genome relatives. These bridging species can be readily crossed with other diploid material expanding the variation associated with the *B. napus* domestication loci. Only once the desired diploid material has been enriched with domestication genes, will we attempt to regenerate synthetic *B. napus* (Figure 2). The newly generated synthetic *B. napus* will possess all of the adaptive traits favourable to the Canadian environment, including spring growth habit, early flowering time, and growth vigour along with the desirable domestication traits that crucially ensures it will be self-compatible and fertile, allowing the genetic potential of these synthetic lines to be evaluated under field conditions.

Overview: This project has developed bridging species for both the A and C genomes that will allow the direct introduction of valuable alleles from diploid Brassica species into the amphidiploid *B. napus* gene pool (Figure 2). This is achieved by first domesticating the diploid Brassica species by introducing *B. napus* alleles that are required for it to be a successful oilseed crop. The introduction of these alleles into both the A- and C- genome backgrounds ensures that any synthetic *B. napus* material developed using this diploid material as parents can be easily evaluated in field conditions as they will be fertile. The crosses used were carefully designed so that the bridging lines combine alleles for early flowering time, spring growth habit and growth vigour so that any resultant synthetic lines are adapted to the Canadian prairie environment. This project has established bridging lines that can be used to introduce new variation into *B. napus* from its crop wild relatives.

2.1 Objectives:

The major goals of this project are the development of new diploid bridging germplasm that is generated by successive backcrossing and marker-assisted selection, followed by the generation of new fertile synthetic *B. napus* using these diploids as parents. Experiments will be conducted to understand and reduce the strength of the genetic block that significantly impedes interspecific crossing efficiency for more direct access to diploid genetic diversity. To

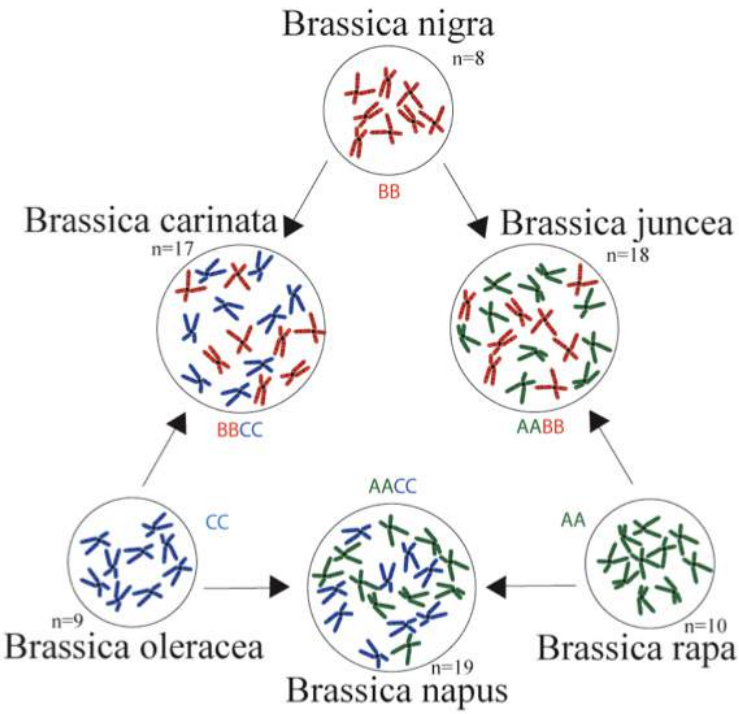

Figure 2: Diagram showing the relative relationship among the six major Brassica species. An interspecific hybridization event between each of the three diploid species has resulted in the generation of three tetraploid species. The species of interest are *B. rapa* (AA), *B. oleracea* (CC) and *B. napus* (AACC).

complement the genetic resources available within the PGRC collection, additional A- and C- genome germplasm will be requested from international germplasm collections.

- Develop A-genome and C-genome bridging lines by introducing key *B. napus* alleles that are adapted to Canadian environments
- Domesticate identified A-genome and C-genome variation, including C-genome variation with new Blackleg resistance by introducing identified *B. napus* alleles
- Generate synthetic *B. napus* germplasm using the domesticated A and C diploid germplasm
- Confirm the successful introgression of blackleg resistance into *B. napus* using disease assays
- Use genome engineering to reduce the impact of key pathways that maintain the genetic block preventing interspecific hybridization

3 Methods

3.1 Strategies to increase the available A- and C-genome variation

This project builds on longstanding research conducted at the SRDC examining the effect of chromosome manipulation and aneuploidy on gene expression. The foundational material generated provided the opportunity to develop Brassica bridging lines possessing domesticated features for both the A-genome and C-genome diploid species. This can be achieved by pursuing the crossing strategy outlined in Figure 3. The diploid germplasm was chosen so that it possessed a spring growth habit and is self-compatible, simplifying the crosses required for their development. The A-genome germplasm (*B. rapa*) was also selected for canola quality characteristics. This material was complemented by C-genome material (*B. oleracea*) identified from a doubled haploid population possessing the combined characteristics of early flowering time, seedling vigour and lacking any vernalization requirement. The amphidiploid line (*B. napus*) was selected from elite canola germplasm adapted to the Canadian environment possessing no known translocation events but lacking blackleg resistance (R) genes. Crosses were made between the amphidiploid (AACC) and each diploid parent (AA & CC) to generate two independent triploid progeny (AAC & ACC). The validity of the triploids was confirmed and the appropriate triploid plant was backcrossed to its diploid parent ([AAC x AA] & [ACC x CC]) to generate segregating backcross (B_1) populations. The B_1 individuals were genotyped using the Brassica 60K SNP array, and markers known to flank the desired alleles were used to identify backcross lines possessing the appropriate amphidiploid (Topas) A-genome or C-genome chromosome regions. Domesticated alleles were selected for early flowering time, self-compatibility and chromosome pairing control. Continued genotyping and marker assisted selection (MAS) for the tetraploid alleles were used throughout successive backcross generations along with simultaneous selection to reduce the number of univalent *B. napus* chromosomes, accelerating the return to the A-genome (2n=20) or C-genome (2n=18) karyotype for each bridging line. The return to the diploid karyotype is necessary before crossing with additional diploid variation. It is anticipated that on average 50% of the univalent *B. napus* chromosomes will be lost at each generation. Therefore, a total of four or five backcross generations will be required to remove all aneuploid chromosomes from the backcross population, returning the material to the chromosome number of the diploid brassica karyotype.

3.1.1 Plant materials

Seeds of the parental lines, diverse diploid and tetraploid lines, triploid plants and the aneuploid populations were grown at the Agriculture and Agri-Food Canada Research Centre, Saskatoon. All plants were grown in 25-cm pots

in the greenhouse at 18°—24°C ± 2 °C with a 16 hour photoperiod. The growth of many of these plants required additional support of tissue culture techniques promoting embryo rescue. Plant material requiring vernalization to promote flowering were transferred to a growth chamber set at 4°C with a 12 hour photoperiod for 12 weeks. Humid growth chambers set at 20°C with a 16 hour photoperiod delivering 175 μMol M^2 $sec^{-}1$ of light were used to promote effective transfer of plants from tissue culture to the greenhouse environment.

3.1.2 Interspecific Hybridization and backcrossing

Interspecific hybridization was performed between Topas x TODH344 and Topas x R-o-18 using the Topas as female parent, to generate triploid plants. The ACC triploid plants were used in backcrosses with TODH334 to produce C-genome B_1 material and the AAC triplod plants were crossed to Reward to produce A-genome B_1 material. Selections were made using molecular markers on these aueuploid individuals and successive rounds of backcrossing to either TODH334 (C-genome) or Reward (A-genome) were conducted. Crosses were made using mature flower buds that were emasculated ~2–3 days prior to reaching anthesis. Emasculation involved the careful removal of anthers using clean forceps. Fresh pollen grains were harvested from freshly opened florets of the male parent and placed on the stigma of emasculated florets. A crossing bag was placed on the pollinated buds to prevent unwanted pollination.

3.1.3 Embryo Rescue and Plant Regeneration

There are significant barriers that prevent successful interspecific hybridization. To overcome these issues, embryo rescue was applied to recover viable plants from dissected embryos following tissue culture. Developing siliques were collected 10-20 days following pollination. The siliques were surface sterilized by treating with 70% ethanol and a 1% sodium hypochlorite solution. The siliques were washed with sterile distilled water under aseptic condition following each treatment. Although the majority of the ovules were aborted, developing ovules containing visible embryos were excised from the silique. Isolated ovules were cultured on Petri-dishes containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 9 g/L agar, pH 5.8. The cultured ovules were maintained under fluorescent light with a 14 h photoperiod at 23°C ± 2 °C to obtain viable embryos after 8–10 days of culture. The rescued embryos continued to grow, forming a shoot within 15–20 days. Initiation of root systems from the sub-cultured shoot tissues were encouraged using basal MS salts. Established plants were carefully transplanted into soil and incubated under reduced humidity to form a functional cuticle before being transferred to the greenhouse to complete their life cycle (Figures 7 and 8).

3.1.4 DNA extraction of qunatification

DNA was prepared for genotyping using the CTAB method. Breifly, freshly collected tissues (~50 mg) were harvested and frozen in liquid nitrogen. Tissues lysis was performed using a mortar and pestle under liquid nitrogen to form a fine powder. A total of 800 μL of warmed 3x CTAB extraction buffer was added to the powdered tissues in the mortar and mixed before being transferred to a microcentrifuge tube and incubated at 65°C for 1 hour. DNA was isolated by adding an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and the organic aqueous phase separated by centrifugation at 26,500 x g for 15 minutes. The aqueous phase was transferred to a new micocentrifuge tube to which 50% volume of 6M NaCl, 10% volume of 3M potassium acetate and 66% volume of isopropyl alcohol was added to precipitate DNA. The DNA was pelleted using centrifugation at 26,500 x g for 15 minutes and washed using 70% ethanol before being resuspended in 1x TE buffer (Doyle & Doyle, 1987; and Cullings 1992).

3.1.5 Kompetitive Allele Specific PCR

KASP (Kompetitive Allele Specific PCR) genotyping was first performed on all individuals that survived tissue culture protocols supporting embryo rescue. Genotyping determined those individuals possessing the desired Topas alleles in the regions of interest for the A or C genome. Primers to the regions of interest were designed using the Primer Picker program and sequence data from target regions of the *B. napus*, *B. rapa* and *B. oleracea* reference genomes. The KASP strategy uses allele specific forward primers with unique tail sequences and amplifies in a chain reaction using common reverse primer. KASP allele identification uses FAM and HEX labelled oligos able to bind to the tail sequences on the forward primers. The samples are amplified using PCR that results in products with either the fluorescent FAM or HEX labels. Following PCR, the fluorescence in each sample is read to determine the genotype of each line (Figure 11). Each backcross individual will either be homozygous for the diploid allele (0/0) or heterozygous containing a diploid and tetraploid allele (1/0). Those heterozygous individuals (contained the Topas allele) at any of the regions of interest were selected for further crossing.

3.1.6 Illumina Infinium SNP array

The Illumina Infinium SNP array contains a total of 90K SNP loci. This technology allows comprehensive genotyping throughout the genome of Brassica plants affording simultaneous analysis of the regions of interest and determining the univalent chromosomal content from the C genome or A genome present in each line. These data provide an assessment on the extent of aneuploidy in each backcross individual. The *B. napus* 60K Illumina Infinium SNP array for genotyping and the Brassica 90K Illumina Infinium SNP array were used to genotype individuals in this project. Extracted DNA was hybridized to the Infinium array following the manufacturer's instructions. The hybridized arrays were scanned using the Illumina HiScan and the SNP data was analyzed using the genotyping module of the Illumina GenomeStudio software package. A two dimensional image of each SNP marker is produced, graphing the position of each individual based on fluorescent intensity. The Illumina GenomeStudio software is designed for diploid species and therefore can not be used to score two loci simultaneously (Figure 9). Due to the polyploid nature of *B. napus* and the propensity for allopolyploids to undergo homoeologous recombination, each polymorphic loci (around 20,000) underwent the painstaking task of visual inspection to determine the correct genotype assignment. Individuals that possessed Topas alleles for any region of interest were subjected to more backcrossing. Preference was provided to those individuals possessing a reduced number of aneuploid chromosomes where possible.

3.1.7 Plant transformation

B. oleracea transormation was conducted using a *A. tumefaciens* mediated approach using 4-day-old *B. oleracea* cotyledon explants from the doubled haploid genotype TO1000DH3. Selection for transformed tissue was performed using kanamycin with a transformation efficiency ~10-20%.

3.1.8 Gene editing

Transgenic *B. oleracea* plants were developed carrying guide RNA to five key genes identified as playing a role in hybridization control. These were DRD1, DRD2, SUVH9, RDR2 and MEDEA. The guide RNA were cloned into a transformation vector based on the Golden-Gate strategy and transformed into TO1000DH3. The guide RNA causes gene editing at the target allele, which can be segregated away from the insertion of the transformation vector.

4 Results

4.1 Diploid bridging line development

The premise of the bridging line strategy is to domesticate diploid brassica germplasm using key *B. napus* alleles to ensure that any new synthetic tetraploid germplasm stemming from the bridged diploid material will be fertile, self-compatible and available for agronomic characterized without further intervention. The *B. napus* alleles required for this are found at two loci in each diploid genome, one controlling the abilty to receive self pollen and the other to limit homoeologous chromosome pairing and therefore the fidelity of meiosis in a tetraploid state (Kitashiba and Nasrallah 2014; Higgins *et al.*, 2020).
The constrains provided by the Canadian environment requiring successful canola varieties to flower early, exhibit a spring-type (annual) growth habit with strong seedling vigour and a robust stem to prevent lodging. Some of these traits are readily available in *B. rapa* germplam but *B. oleracea* are usually biennial and have a late flowering time complicating their use in Canadian breeding programs.

4.1.1 Identification of optimal diploid germplasm

The constraints applied by the Canadian environment are unique and provide challenges for the development of domesticated diploid lines. Any new material needs to be able to be rapidly evaluated in Canadian field conditions. If successful, the introduction of new domesticated diploid material can be combined to form populations of new *B. napus* material through the production of synthetic tetraploid using established techniques that promote chromosome doubling following interspecific hybridization.

4.1.1.1 A-genome material The selection of A-genome diploid material for use in the development of the triploid lines and subsequent backcrossing was straight forward. Material was received from the former AAFC *B. rapa* breeding program that possessed homozygous alleles at loci controlling traits including annual growth habit, early flowering time and low glucosinolyate levels (Faulk personal communication). The *B. rapa* line R-o-18 was crossed to Topas to form successful triploid germplasm and Reward was used for successive backcrossing (Figure 5). These lines were selected as they are self-compatible, minimizing the probability of including modifying loci that might lead to an unanticipated self-incompatible response.

4.1.1.2 C-genome material The selection of C-genome diploid material for use in the development of the triploid lines and subsequent backcrossing was not straight forward. The majority of *B. oleracea* material is biennial requiring three months of low temperature exposure to satisfy their vernalization requirement. To avoid these complications *B. oleracea* germplasm was selected from a doubled-haploid population derived from a cross between TO1000DH3 (wild kale) and Early Big (broccoli) (Iniguez-Luy *et al.*, 2008; 2009). The resulting TODH population was assessed to identify an annual individual with strong seedling vigour, robust stem growth and possessing an early flowering time, focusing on an optimal line TODH344 (Robinson unpublished). The *B. oleracea* line TODH344 was crossed to Topas to form successful triploid germplasm and the selected TODH344 line was used for successive backcrossing (Figure 4).

4.1.2 The challenge with bridging line development

In order to develop the ideal domesticated bridging lines possessing alleles for fertility, self-compatibility, seedling vigour, early flowering time and annual growth habit, alleles at a minimum of five loci need to be selected. The major loci controlling these traits have been mapped (Kitashiba and Nasrallah 2014; Higgins *et al.* 2020, Robinson unpublished; Rahmon *et al.*, 2013; Rahmon *et al.*, 2015; Schiessl *et al.* 2019) and the use of a unified comprehensive marker framework utilizing key reference Brassica genomic resources (Parkin *et al.*, 2014; https:

//www.ncbi.nlm.nih.gov/datasets/genome/GCA_017639395.1/) make manipulation of these characters practicable (Clarke *et al.*, 2016).

There are challenges to enriching genetic diversity in spring *B. napus* using diploid germplasm, this results primarily from the low fertility levels (Rahman *et al.*, 2015) due to aneuploidy and other hybridization barriers that prevent viable seed production (Köhler *et al.*, 2010). The reduction in fertility is observed in both the A- and C- genome crosses, but the C-genome barrier is profound where no viable seed is recovered. To overcome these impediments, the developing embryos need to be excised and rescued using tissue culture techniques. This means that population sizes are severely restricted in all of the backcross populations, particularly so for the C-genome crosses. Typically, the population size of the embryo rescued plants in a backcross population will be less than 30 individuals.

The probability of finding the required individual for successive rounds of backcrossing is a function of the number of alleles segregating at each locus (Figures 3 and 4). When there are three loci being selected for, with segregation of two alleles at each locus the desired genotype occurs at a frequency of 1/32 in a backcross. The number of plants required to be analyzed to find the desired genotype is 94 or 145 with a 95% or 99% probability respectively. The problem of selecting for increasing numbers of segregating loci is presented in Figure 3. The probability of obtaining the desired genotype with a restricted population size in a backcross population is presented in Table 1. These estimates are based on the assumption of no segregation bias for either of the two alleles.

The number of individuals required to be examined to recover the desired genotype with a 99% probability for a single segregating loci is seven. When the number of segregating loci increases to two, the population size required to be screened increases to 35 individuals. Once the number of loci being selected increases to three or above, population sizes greater than 100 are required to recover the desired genotype. Screening populations consisting of 100 individuals, provides a ~95%, ~54% or ~18% probability of recovering the desired genotype (Table 1). These parameters highlight the challenge being undertaken during this project.

These calculations highlight that managing two or three loci is all that can be realistically achieved with the restricted population sizes recovered through embryo rescue. The desired genotype was reduced to consider only two segregating loci, those responsible for controlling fertility and self-compatibility. The remaining traits were managed by selecting germplasm that does not segregate for alleles at those loci. There are early flowering time alleles, annual habit alleles and seed vigour present in Topas, TODH344 and Reward, meaning that any trait segregation for these traits will be minimal.

Table 1: The probability of obtaining the desired genotype with different numbers of backcross segregating alleles. The probabilty of achieving the event at 95% level is provided for one locus (P1L) through five loci (P5L) assuming no bias in allele transmission.

N	P1L	P2L	P3L	P4L	P5L
1	0.500	0.125	0.031	0.008	0.002
5	0.969	0.487	0.147	0.038	0.010
10	0.999	0.737	0.272	0.075	0.019
15	1.000	0.865	0.379	0.111	0.029
20	1.000	0.931	0.470	0.145	0.038
25	1.000	0.965	0.548	0.178	0.048
30	1.000	0.982	0.614	0.210	0.057
35	1.000	0.991	0.671	0.240	0.066
40	1.000	0.995	0.719	0.269	0.075
45	1.000	0.998	0.760	0.297	0.084

N	P1L	P2L	P3L	P4L	P5L
47	1.000	0.998	0.775	0.308	0.088
71	1.000	1.000	0.895	0.427	0.130
100	1.000	1.000	0.958	0.544	0.178

4.1.3 Towards the development of the bridging lines

4.1.3.1 Generation of triploid material The bridging line strategy started by crossing [Topas x R-o-18] and [Topas x TODH344] to form triploid material. The A-genome ([Topas x R-o-18]) triploid (AAC) possessed 29 chromosomes and the C-genome ([Topas x TODH344]) triploid (ACC) possessed 28 chromosomes. Successful seed was obtained with a little effort with the details listed in Table 2. The genome size and fidelity of the triploids was confirmed using flow cytrometry and genotyping using the Brassica SNP array. A single triploid plant was used in further crosses ([Topas x R-o-18] x [Reward] & [Topas x TODH344] x [TODH344]) to generate the successive generations of backcross populations using Reward and TODH344 as the recurrent diploid A- and C-genome parent, respectively (Figure 3).

Table 2: A pilot study was performed to assess the optimal genotype to use with a focus on those selected sue to the availableity of genomic sequence information.

Cross	No. of buds	No. of seed	Seed per bud
DH12075_x_R_o_18	11	1	0.0909091
DH12075_x_EarlyBig	10	0	0.0000000
DH12075_x_TO1000	12	3	0.2500000
DH12075_x_Reward	9	100	11.1111111
DH12075_x_080798EM_219	11	30	2.7272727
DH12075_x_060DH17	10	1	0.1000000
DH12075_x_NI100	6	1	0.1666667
Topas_x_R_o_18	7	30	4.2857143
Topas_x_EarlyBig	9	0	0.0000000
Topas_x_TO1000	14	3	0.2142857
Topas_x_Reward	17	100	5.8823529
Topas_x_080798EM_219	8	30	3.7500000
Topas_x_060DH17	12	100	8.3333333
Topas_x_NI100	10	4	0.4000000
Rainbow_x_R_o_18	12	1	0.0833333
Rainbow_x_EarlyBig	11	0	0.0000000
Rainbow_x_TO1000	11	0	0.0000000
Rainbow_x_Reward	12	50	4.1666667
Rainbow_x_080798EM_219	9	5	0.5555556
Rainbow_x_060DH17	12	30	2.5000000
Rainbow_x_NI100	13	0	0.0000000

Table 3: Summary of the number of buds crossed, number of seed obtained, number of seed per bud and the resulting chromosome number in the generation of triploid material. These data are not independent (X-squared = 4.7155, df = 1, p-value = 0.02989) indicating there are greater barriers to the formation of triploid C-genome rather than A-genome material.

Cross	No. of buds	No. of seed	Seed per bud	No. of chromosomes
AACC_x_AA	22	8	0.3636364	29
AACC_x_CC	192	23	0.1197917	28

4.1.3.2 Backcross generations As anticipated greater effort was required to generate triploid material using [AACC x CC] crosses rather than [AACC x AA] crosses (Table 3). The quality of the resulting [AAC] triploid plants was greater than the [ACC] triploid plants with many [ACC] triploids producing low amounts of poor quality pollen grains. There are different strategies for utilizing germplasm from crop wild relatives once the triploid plants have been formed. Crosses can be made between triploid and diploid or triploid and tetraploid material leading to introgression of chromosome regions originating from the diploid material. To quantify the differences in efficiency for crosses between ploidy and the triploid lines on both the A- and C-genome, reciprocal crosses were made using either diploid [(AA) or (CC)] material or tetraploid (AACC) material with the appropriate triploid material. As anticipated these data revealed asymmetric responses in the development of backcross populations involving different ploidy-level parents. The data from the A-genome crosses are presented in Table 4 and C-genome on Table 5. A representation of how the total allelc compliment is recoreded is presented in Figure 10 where each locus records the presence of diploid or tetraploid alleles.

4.1.4 Examining these resources reveals asymetric genome responses

The greatest difference was observed when comparing between the A-genome and C-genome crosses using the appropriate triploid. Although viable seed was difficult to generate, with ~1.5 viable seeds being produced per bud crossed using A-genome material, there were profound barriers preventing the production of viable seed using C-genome material where no viable seed could be generated.

Comparisons within the triploid material, comparing the use of diploid and tetraploid material in crosses with the triploids revealed that greater success was observed for viable seed generation when the triploid was used as the female parent, suggesting that pollen viability impaired for the triploid plants (Table 4). A similar phenomenon was observed using C-genome material. The majority of the ACC triploids produced poor pollen or were sterile. The most productive triploid individual was able to produce viable seed when crossed to the tetraploid parent DH12075 but unable to produce seed with diploid C-genome material. The profound hybridization barriers for the diploid material (Table 5), required for bridging line development, could only be overcome using embryo rescue (Figures 7 and 8).

4.1.5 A-genome

Table 4: Summary of the number of buds crossed, number of seed obtained, number of seed per bud in the generation of the A-genome B1 generation. There was a difference in the ability to recover seed dependent on whether the triploid plant was used as the female or male parent (X-squared = 135.31, df = 3, p-value < 2.2e-16).

Cross	No. of buds	No. of seed	Seed per bud
Triploid_x_Reward	71	118	1.6619718
Triploid_x_DH12075	56	90	1.6071429

Cross	No. of buds	No. of seed	Seed per bud
Reward_x_Triploid	231	70	0.3030303
DH12075_x_Triploid	102	18	0.1764706

4.1.6 C-genome

Table 5: Summary of the number of buds crossed, number of seed obtained, number of seed per bud in the generation of the C-genome B1 generation. These data are no independent (X-squared = 91.001, df = 3, p-value < 2.2e-16). There was a difference in the ability to recover seed dependent on whether the triploid plant was used as the female or male parent (X-squared = 33.838, df = 1, p-value = 5.989e-09) with a preference for using the triploid as female parent. Additionally, there was a profound difference in the amount of seed recovered based on the ploidy of the parents (X-squared = 74.492, df = 1, p-value < 2.2e-16), with a preference for using triploid x tetraploid material.

Cross	No. of buds	No. of seed	Seed per bud
Triploid_x_TODH334	95	1	0.0105263
Triploid_x_DH12075	38	15	0.3947368
TODH334_x_Triploid	34	0	0.0000000
DH12075_x_Triploid	43	55	1.2790698

These comparison of C-genome material was not restricted to a single individual. The numbers presented in Table 6 are from crosses using the triploid #4 (Topas x TODH344) and TODH334. A total of nine individual triploid plants were examined using a total of 1463 bud pollinations that delivered zero viable seed when crossed with TODH344.

It was apparent for the success of this project that the major focus of this project needs to be dedicated towards the C-genome line development.

4.1.6.0.1 Strategy for the C-genome The greatest challenge for this project is the generation of the C-genome bridging lines due to the strength of the triploid block. This meant that the majority of the resources for this project were dedicated to solving this problem. A summary of the progress made is presented in Table 6. A total of 4,851 buds were crossed over four backcross generations that yielded 439 rescued embryos resulting in 214 plants. The surviving plants were genotyped to identify the presence of the desired Topas alleles and to determine the presence of aneuploid chromosomes. The early generations of backcrossing produced a lower number of embryos per cross and it was anticipated that fertility would be recovered with the restoration to a *B. oleracea* karyotype (2n=2x=18). Due to the effects of recombination and independent chromosome assortment at meiosis, there is no guarantee that both of the Topas alleles will be inherited in the same individual. The estimations of required populations size (Figure 3) are useful but there is a 1/20 chance that they don't work at 95% probability level. Furthermore, the estimates assume that both Topas alleles are transmitted through meiosis at the same frequency. The segregation ratios observed suggest that this is not the case with the fertility restoring allele being observed at a lower than anticipated frequency. This complicated the backcrossing strategy and the backcross 3 population required them to be maintained in different lines. The presence of any A-genome aneuploid chromosome was sufficient to cause infertility and there was no observable preferential effect from any aneuploid C-genome chromosome. The removal of A-genome aneuploid chromosomes restored fertility allowing self-seed to be collected from the 2n=2x=18 lines.
This is an important development as the problems of required population size becomes significantly more challenging to recover individuals homozygous for the selected Topas alleles (Figure 10). These required population sizes are

beyond those achievable using embryo rescue and require the production of self-seed.

Table 6: Summary of the number of buds crossed, number of embryos rescued, number of plants recovered per generation for the C-genome line development. There was an increase in the frequency of successful embryos rescued and plant recovered with advancing generations. The most difficult generations were the backcross 1 and 2, possesing the highest number of aneuploid A genome chromosomes. There was a difference in the frequency of generating embyros per bud crossed with the frequency increasing as aneuploidy is reduced over B1-B4 generations (X-squared = 82.643, df = 3, p-value < 2.2e-16). The intervention of embyro rescuse appeared to be independent of the effects of aneuploidy (X-squared = 1.8223, df = 3, p-value = 0.6101).

Generation	No. of buds	No. of embryos	No. of plants	embryos per bud	plants per embryo
B1	1598	128	42	0.0801001	0.3281250
B2	1381	70	30	0.0506879	0.4285714
B3	1307	273	117	0.2088753	0.4285714
B4	565	67	25	0.1185841	0.3731343

4.1.6.0.2 Segregation analysis The profound reduction in fertility observed during the C-genome line development poses definite constraints and affects the number of loci that can be effectively managed. Following successive generations of backcrossing and selection for Topas alleles and against A-genome aneuploid chromosomes returns fertility to the diploid bridging lines. Probability theory can then be employed to guide the planning of future experiments, detailing the population sizes required to manage the segregation of the Topas alleles. These details are presented in Figures 3 and 4. However, these population sizes are dependent on the absence of segregation distortion that might favour the transmission of one parental allele over the other.

Table 7: Summary of the number of Topas alleles on C6 and C7 found among the backcross 1 generation. The genotypic group is indicated as the presence or absence of the Topas allele for C6 and C7, the number of individuals observed with this genotype and their frequency among the population. The C6 (X-squared = 1.6667, df = 1, p-value = 0.1967) and C7 (X-squared = 0.066667, df = 1, p-value = 0.7963) diploid and tetraploid alleles segregate independently in the B1 generation.

group	n	prop
0/0	4	26.7
0/1	1	6.7
1/0	3	20.0
1/1	7	46.7

Table 8: Summary of the number of the Topas alleles on C6 and C7 found among the backcross 2 generation. The genotypic group is indicated as the presence or absence of the Topas allele for C6 and C7, the number of individuals observed with this genotype and their frequency among the population. The C6 diploid and tetraploid alleles segregate independently (X-squared = 0.1, df = 1, p-value = 0.7518) whereas the C7 diploid and tetraploid alleles show a segregation bias (X-squared = 12.1, df = 1, p-value = 0.0005042) for the diploid alleles in the B2 generation.

group	n	prop
0/0	17	42.5
0/1	4	10.0
1/0	14	35.0

group	n	prop
1/1	5	12.5

Table 9: Summary of the number of the Topas alleles on C6 and C7 found among the backcross 3 generation. The genotypic group is indicated as the presence or absence of the Topas allele for C6 and C7, the number of individuals observed with this genotype and their frequency among the population. The C6 (X-squared = 4.2609, df = 1, p-value = 0.039) and C7 (X-squared = 3.1304, df = 1, p-value = 0.07684) diploid and tetraploid alleles segregate independently in the B3 generation.

group	n	prop
0/0	19	41.3
0/1	11	23.9
1/0	10	21.7
1/1	6	13.0

Table 10: Summary of the number of the Topas alleles on C6 and C7 found among the backcross 4 generation. The genotypic group is indicated as the presence or absence of the Topas allele for C6 and C7, the number of individuals observed with this genotype and their frequency among the population. The C6 diploid and tetraploid alleles segregate independently (X-squared = 1.8028, df = 1, p-value = 0.1794) whereas the C7 diploid and tetraploid alleles show a segregation bias (X-squared = 76.169, df = 1, p-value < 2.2e-16) for the diploid alleles in the B4 generation.

group	n	prop
0/0	65	45.8
0/1	14	9.9
1/0	58	40.8
1/1	5	3.5

4.1.6.0.3 Strategy for the A-genome The generation of A-genome bridging lines is difficult but is not as challenging as the C-genome bridging lines. Resources were dedicated to the A-genome line development after the the C-genome lines were started and space requirements represented a constraint preventing both populations development from running concurrently. The A-genome line development suffered during the Covid-19 closure as the C-genome material was given the highest priority. The A-genome line development was scaled back upon reopening as resources focused of the more challenging C-genome line development. Fortunately, their greater fertility meant material could be stored as seed, whereas the C-genome material needed to be kept as clones to prevent against further losses. This increased the C-genome line space requirement. Crossed were made to generate backcross generations. These plants were genotyped to identify the presence of the desired Topas alleles and to determine the presence of aneuploid C-genome chromosomes. The discontinuation of the Illumina 90K SNP genotyping array prevented comprehensive genotyping, slowing selection. The early generations produced seed and it was anticipated that full fertility would be recovered with the restoration to a *B. rapa* karyotype (2n=2x=20). The extent of the triploid block on the A-genome side of the crossing strategy is not as strong as the block on the C-genome side of the crossing strategy. Additionally, although the fertility of the A-genome backcross material was significantly reduced, some self-seed was set in lines possessing aneuploid C-genome chromosomes.

This additional fertility was welcome, as the number of desired Topas loci being manipulated for the A-genome

is three. One locus controlling self-compatibility and two loci controlling fertility, but with uneven contributions. Nevertheless, to be most effective as bridging lines, all three loci will be required.

Similar to the results observed for the C-genome line development, the A-genome line development necessitated the maintenance of the Topas alleles in separate individuals due to the segregation of the three loci within the backcross populations. Successive generations of backcrossing led to the reduction of aneuploid C-genome chromosomes, losing on average, 50% of them at each generation. Therefore it was anticipated to take four to five backcross generation to remove them with out selection. Where available, the use of SNP genotyping to identify the presence of aneuploid C-genome chromosomes meant that marker assisted selection (MAS) could be used with selection for lines containing the desired Topas domestication alleles taking precedence. This constraint was unfavourable for the A-genome bridging line development with those lines possessing the desired Topas alleles also containing a higher than anticipated number of C-genome aneuploid chromosomes. Fortunately, the reverse situation was observed during the development of the C-genome bridging lines where rapid selection again aneuploid A-genome chromosomes was possible.

Table 11: Summary of the number of buds crossed, number of embryos rescued, number of plants recovered per generation for the A-genome line development. There was an increase in the frequency of successful embryos rescued and plant recovered with advancing generations. The most difficult generations were the backcross 1 and 2, possesing the highest number of aneuploid A genome chromosomes.

Cross	No. of buds	No. of plants used	No. of seed	No. sterile plants	Seed per bud
Triploid_x_Reward	100	20	10	19	0.100000
Triploid_x_DH12075	150	31	1560	2	10.400000
Reward_x_Triploid	110	22	177	14	1.609091
DH12075_x_Triploid	65	15	690	1	10.615385

Table 12: Summary of the number of buds crossed, number of embryos rescued, number of plants in the first backcross generation for the A-genome line development. There was sufficent seed developed without the need for embryo rescued and the number of seed recovered advanced with successive generations. The number of buds crossed and seed recovered was not counted as it became less challenging. The most difficult generations were the backcross 1 and 2, possesing the highest number of aneuploid C genome chromosomes.

Cross	No. of buds	No. of plants used	No. of seed	No. sterile plants	Seed per bud
B1_x_Rainbow	441	441	909	909	2.0612245
B1_x_DH12075	154	154	458	458	2.9740260
B1_x_Reward	226	226	186	186	0.8230088

4.1.6.1 Challenges during the project One of the greatest challenges experienced during this project was the necessary adjustments resulting from the Covid-19 pandemic. AAFC restricted access to the Research Centre and the building closure resulted in both a loss of time and more significantly germplasm losses. This most seriously affected the developed of the bridging lines as unfortunately some of the most advanced lines were lost during tissue culture and transfer from tissue culture to a soil substrate. The high risk associated with C-genome line development meant it received priority and the expense of the A-genome line development. With the completion of the C-genome bridging lines the A-genome line will catch up rapidly due the absence of a tissue culture phase in the line development.

4.1.6.2 Current status of bridging line development The bridging line development is now at the stage where we have managed to return the C-genome lines to the *B. oleracea* karyotype and fertility has been restored in these diploid lines. Due to the constraints imposed by population size, an additional generation is required to combine both the desired *B. napus* alleles into the same genetic background. The increased fertility will speed up the transfer of these alleles into the collected C- genome diversity obtained through collaboration with the PGRC as there is no longer an absolute requirement for embryo rescue to recover viable plants.

The A-genome lines are slightly behind the C-genome line development but their enhanced fertility is favourable, enabling this material to catch up, the only constraint being available growth space. The enhanced fertility observed among the A- genome material allowed an additional (third) locus to be introgressed, at a cost of briefly slowing line development. As we move into March 2024, genotyping of the next generation should identify the lines that will be used to cross into the diversity that has been established as part of this project. These crosses are anticipated to occur during the fall of 2024 but the challenges of synchronizing flowering time between the bridging lines and the biennial diversity will pose its own management challenges. Diversity crosses will be made for the C-genome bridging lines while the A-genome bridging lines are completed.

The remaining task will be the analysis of large enough populations to recover the desired homozygous diploid material before making synthetic *B. napus*. It is anticipated that additional SNP genotyping resources will become available in 2025 that are needed to support future comprehensive genotyping.

4.1.6.3 Population sizes need to be increased to recover homozygous domestication alleles It is important for the success of the project that fertility and seed set be returned to those levels observed in the diploid parents (Reward and TODH344) to enable the identification of bridging lines homozygous of the Topas domestication alleles. The necessity to make dihybrid (C-genome) and trihybrid (A-genome) crosses will require population sizes of 72 and 293 respectively to be 99% confident of finding the desired individual. These numbers exceed the capacity of using embryo rescue. Further, this process will need to be repeated following the introduction of the domestication alleles into selected diverse wild relatives. It is therefore, worth the investment in generating the best possible bridging lines possessing high levels of fecundity.

Table 13: The probability of obtaining the desired homozygous genotype with different numbers of segregating alleles. The probabilty of achieving the event at 95% level is provided for one locus (P1L) through five loci (P5L) assuming no bias in allele transmission.

N	P1L	P2L	P3L	P4L	P5L
1	0.250	0.062	0.016	0.004	0.001
5	0.763	0.276	0.076	0.019	0.005
10	0.944	0.476	0.146	0.038	0.010
15	0.987	0.620	0.210	0.057	0.015
20	0.997	0.725	0.270	0.075	0.019
25	0.999	0.801	0.325	0.093	0.024
30	1.000	0.856	0.377	0.111	0.029
35	1.000	0.896	0.424	0.128	0.034
40	1.000	0.924	0.467	0.145	0.038
45	1.000	0.945	0.508	0.161	0.043
47	1.000	0.952	0.523	0.168	0.045
71	1.000	0.990	0.673	0.243	0.067
100	1.000	0.998	0.793	0.324	0.093

4.2 Identification of new diploid genetic diversity

The search for additional variation to complement that stored in the Plant Genetic Resources of Canada (PGRC) was conducted. This is a difficult and challenging task as access to to information regarding Brassica and wider Brassicaceae collections remains cryptic. Additionally, there are considerable difficulties maintaining and distributing these resources due to challenges stemming from vernaliaztion requirements and self-incompatibly among these species, reducing seed set and thus seed availability.

During this project, contact was made to the Italian GeneBank and the Spanish GeneBank where collections of Brassicacae material was held. Successful contact with representatives in Spain and successful requests for germplasm were been placed. However, contact with representatives in Italy were unsuccessful where very limited seed was held in their collection preventing seed distribution.
A total of nine lines were requested from the Spanish collection to complement those variation already held at the PGRC in Saskatoon. Rigorous selection was applied by PRGC staff to minimize the possibility of requesting duplicated material housed a the Saskatoon GeneBank. A list of the resources requested is presented in Table 4. These resources include two A-genome (*B. rapa*) and seven C-genome (*B. oleracea*) representatives. Among these the more distantly related species will likely be more challenging to introduce their variation through hybridization. Import permits were obtained supporting seed acquisition. Correspondence with the Spanish GeneBank indicated a lack of resources to process the request immediately and although delays were caused by Covid-19 precautions, the seed was successfully received. A representation of the geographic area of the seed obtained are highlighted in Figures 12 and 13.

Table 14: List of germplasm examined using the Canola Domestication Array. There is a necessary focus on C-genome varaition, particularly within *B. oleracea*.

Species	Count
B. oleracea	44
B. rapa	4
B. tournefortii	2
B. napus	2
B. incana	1
B. montana	1
B. villosa	1
B. bourgeaui	1
B. rupestris	1
B. hilarionis	1

There are nine new requests among the Spanish collection. Five of the lines are *B. oleracea* (2n=2x=18); two are *B. repanda* (2n=2x=20); with single representative of both *B. oxyrrhina* (2n=2x=18) and *B. bourgeaui* (2n=2x=18). The species *B. oxyrrhina* and *B. bourgeaui* add to the diversity for the C-genome, whereas *B. repanda* adds to increase the diversity in the A-genome. The origin of the lines is presented graphically as maps where lines were selected from environments where alleles might evolve to withstand abiotic stresses (Figures 12 & 13).

Additional Brassica diploid material was sourced from the United Kingdom (University of Warwick). A total of twenty *B. oleracea* lines were received increasing our available C-genome diversity. The AAFC breeding collection was examined for additional variation where a total of twelve Brassica wild relatives were identified. These included

six accessions of *B. tournefortii* (2n=2x=20) and one accession each from *B. adpressa*, *B. amarifolia*, *B. barrelieri*, *B. cheiranthos*, *B. cretica*, *B. fruticulosa*, *B. gravinae*, *B. incana*, *B. maurorum*, *B. oxyrrhina* and *B. villosa* (2n=2x=18). These lines are unencumbered by germplasm treaty restrictions and offer the optimal materila for future *B. napus* genetic improvement (Table 15).

Representatives from these collections were grown out and characterized under greenhouse conditions. Unfortunately, many lines were not viable. Where seed germinated, self-seed was retained where self-incompatibility was not strong. In those instances where self-seed was not available, crosses with made with TO1000 and TODH344 to retain the viability of some of the variation. To extract maximal value, these collected resources need to be evaluated for useful phenotypic variation that can be directly accessed for future *B. napus* improvement. Additionally, the absolute genetic variation can be exploited to improve heterotic potential in the formation of new hybrids.

To demonstrate the potential of the diploid bridging lines, in the first instance, new diploid diversity already within *B. rapa* and *B. oleracea* gene pool will be selected for domestication. These include material identified by possessing resistance alleles to blackleg and clubroot pathogens. This eases their hybridization to the bridging lines considerably and offers no problems other than synchronizing flowering time for crossing. Crosses with the more diverse wild relatives will follow, selecting first those species most amenable to hybridization.

Table 15: List of germplasm obtained from the AAFC germplasm collection. These lines represent unincumbered material that can be readily introduced in the B. napus germplasm using the diploid bridging strategy.

Species	Count
B. oleracea	20
B. tournefortii	6
B. adpressa	1
B. amarifolia	1
B. barrelieri	1
B. cheiranthos	1
B. cretica	1
B. fruticulosa	1
B. gravinae	1
B. incana	1
B. maurorum	1
B. oxyrrhina	1
B. villosa	1

Table 16: Summary of progress for CRISPR mutations in *B. oleracea*.

Species	Gene	Guide.design	Transformation	T.DNA	Mutated.allele
B. oleracea	DRD1	yes	yes	yes	yes
B. oleracea	DRD2	yes	yes	yes	yes
B. oleracea	SUVH9	yes	yes	yes	yes
B. oleracea	RDR2	yes	yes	yes	yes
B. oleracea	MEDEA	yes	yes	yes	yes

4.2.0.1 Identification of Blackleg resistant material Seed was provided by Dr. Borhan that was previously identified as possessing a new source of blackleg resistance (*Leptosperia maculans*). This material was identified by Drs. Borhan and Larkan and is the primary variation to be introduced using the bridging lines, demonstrating their utility. We await the molecular marker information from Drs. Borhan and Larkan to follow the blackleg resistance loci in crosses. The material provided has a strong vernalization requirement that will complicate its combination with its bridging line.

Genetic marker information is not available from Dr. Borhan and the career development of Dr. Larkan has seen him move from oilseed to pulse focused research. This has reduced the capacity of research available to characterize the new material. Crosses have been made between the putative blackleg resistant line and a susceptible parent to develop F_1 individuals that can be used to make mapping populations. These resources will be necessary for the introgresion of the blackleg resistance alleles into the bridging lines. The line provided will be crossed to the *B. oleracea* bridging line once a single line possessing both the *B. napus* alleles on C6 and C7 has been generated.

4.2.0.2 Identification of Clubroot resistant material Clubroot disease (*Plasmodiophora brassicae*) is major threat to canola production throughout the prairies. Root infection results in the production of galls (swollen tissue) that reduces water and nutrients uptake causing yield loss. *Plasmodiophora brassicae* possess a significant challenge due to its ability to survive in the soil as resting spores that are viable for up to 20 years with a half-life of two to four years. Resting spores produce zoospores with flagella that enable them to travel short distances and infect root hairs. The ability of *P. brassicae* to infect related weed species further complicates pathogen control. *P. brassicae* is able to infect stinkweed (*Thlaspi arvense*), Shepherd's purse (*Capsella bursa-pastoris*), flixweed (*Descurainia sophia*) as well as species in the mustard family. There is a need for additional sources of clubroot resistance and the introduction of resistance alleles from diploid wild-relatives offers an attractive prospect. The collection of diploid Brassica genetic resources at AAFC includes A-genome germplasm identified as clubroot resistance. Currently, it remains unknown if this source is allelic with previously identified sources available to canola breeders or whether this represents a new resource source. Nevertheless, this A-genome wild relative of canola represents an excellent source of variation to demonstrate the potential of the A-genome bridging material. The material identified has a strong vernalization requirement that will complicate its combination with the bridging line.

The Brassica genetic resources assembled by this project are valuable and together with the development of the diploid bridging lines, their accessibility to canola breeders has taken a giant leap forward.

4.2.1 Generate RNASeq from pollen samples to identify targets for genome editing

The critical goal of the project is to develop new material that promotes the introgression of alleles from diploid species (*B. rapa* & *B. oleracea*) into the tetraploid species (*B. napus*). There are significant barriers that prevent the success of interspecific hybridizations and contribute to the triploid block. The crosses are restricted by genes expressed in the pollen cells that prevent successful hybridization. The triploid block can be relieved by identifying these factors in pollen and making specific changes to reduce them through the generation of null-alleles in these genes using CRISPR. These factors are epigenetic factors controlling paternal imprinting and will be identified from assessing the small RNA complements in pollen. Progress to date has seen the isolation of small RNA from the *B. oleracea* line TO1000, sequencing libraries have been generated and small RNA molecules sequenced. These data will be interrogated to identify pollen-specific miRNA species as well as enriched populations mapping close to known imprinted genes. These loci will become targets of the gene editing approach.

4.2.2 Generate mutations in target genes using CrispR genome editing.

The methodology to generate targeted CRISPR mutation in *B. oleracea* has been established during this project using the *B. oleracea* line (TO1000) (Figure 11). These strategies can be applied to targets identified from the small RNA libraries from pollen. Establishment of methods for *B. oleracea* transformation to deliver CRISPR-CAS9 containing T-DNA with guide RNA was demonstrated by inducing mutations in gene targets known to be involved in paternal imprinting. The progress of this research is presented in Table 16. The project has produced CRISPR constructs targeting the genes homologous to *DRD1*, *RDR2*, *SUVH9* and *MEDEA* in *A. thaliana*. We have successfully transformed DH1012 with constructs targeting *DRD1* and *RDR2* and regenerated transgenic plants using the strategy presented in Figure 14. We have confirmed the presence of mutations in the targeted genes in the transgenic lines. Future research will focus on completing transformation of *SUVH9* and *MEDEA* targeting constructs and backcrossing mutated plants to DH1012 to eliminate transgene and potential somaclonal mutations (Figure 15).

Our objective is to use gene editing to reduce the reproductive barriers that prevent hybridization between *Brassica oleracea* and *Brassica rapa*. Based on study of the model brassicaceae *Arabidopsis thaliana*, we hypothesized that mutations in the epigenetic factors *DRD1* and *RDR2* in *B. oleracea* could help make successful hybridization possible. Using the CRISPR/Cas9 system, we were able to produce and isolate a mutant line for each of the selected genes. We will now proceed with crossing these lines with *B. rapa* lines to test their effect on inter-specific crosses. In parallel, we have also performed small RNA sequencing on the pollen of *B. oleracea*. We will now proceed with the analysis of that data in order to identify new potential targets for gene editing. Previous studies have demonstrated that the small RNA load in pollen can have an influence on inter-ploidy crosses but the effect on inter-species crosses has never been tested. We plan to engineer the small RNA content in order to test that approach in future experiments. An example on the *DRD1* mutant is provided in Figure 15 where the identification of plants possessing homozygous and heterozygous mutant alleles has been determined through RFLP genotyping (A). The morphological characteristics of the homozygous *drd1* line are immediately apparent and wild-type characteristics are recovered in heterozygous individuals.

5 Conclusions

The canola quality spring oilseed *Brassica napus* L. is widely grown in the Prairie Provinces of Alberta, Manitoba and Saskatchewan and is one of Canada's most important crop species. However, like many recent polyploid species, the narrow genetic diversity in spring *B. napus* needs to be broadened to meet future breeding goals as genetic diversity is critical for developing new cultivars. This is most readily achieved by enriching its C-genome with diversity present in its progenitor species *B. oleracea* L.
However this is no small task. There are significant barriers to hybridization that have led to the isolation of the *B. napus* C-genome. Similar barriers exist for the A-genome, but not to the same extent as exhibited by comparing *B. napus* A- and C- genome linkage disequilibrium (Ebersbach et al., 2022). Methods to increase diversity by generating synthetic *B. napus* lines are hampered by the introduction in the progeny of self-incompatibility, reductions in fertility and the introduction of vernalization requirements and late flowering alleles. These conspire to prevent the assessment of the new variation, as good seed set is required to evaluate yield in canola.

The establishment of the Brassica diploid bridging species to access diversity contained in Brassica wild relatives represents a novel valuable innovation. The judicious selection of germplasm ensures that new tetraploid Brassica resulting from the combination of these bridging lines will be adapted to the Canadian environment. This immediately makes the new variation accessible for evaluation in the field.

New *B. napus* variation can now be generated by crossing selected *B. rapa*, *B. oleracea* and wild relative germplasm following the introduction of the domestication alleles originally derived from Topas (Figures 5 & 6). The resulting allohaploid progeny will be treated with colchicine, a chemical agent that interferes with spindle fiber formation preventing chromosome disjunction. Following treatment with colchicine, the resultant chromosome doubling leads to the formation of an amphidiploid (AACC) plant, reestablishing fertility from the initial infertile allohaploid (AC) plant. The karyotype of the synthetic *B. napus* plants will be confirmed using ploidy analysis ane genotyping. Seed will be multiplied and the material will then be available for characterization. The primary goal of this project was to establish a method to transfer valable diploid alleles. However, similar to other allopolyploid species, the resulting material will possess fixed heterosis and the diversity contained in the newly generated material can perhaps form the basis of future heterotic pools should it be sufficiently diverse and able to be used in breeding programs.

5.1 Recommendations

Capitalize on the investment of developing these unique genetic resources by:

- **Finalize the optimal diploid bridging material possessing the greatest seed set**
- **Cross a selection of the diverse wild relatives collected to the bridging lines**
- **Generate synthetic *B. napus* germplasm using the new domesticated A and C diploid germplasm**
- **Assess the new synthetic *B. napus* germplasm in field trials**
- **Distribute the *B. napus* diversity into breeding programs**

6 Acknowledgements

The funding sources supporting this project have been communicated in all aspects of the research project. Funding sources are acknowledged in internal and external communications for research papers, presentations and conference proceedings. We are grateful to Agriculture and Agri-Food Canada for GRDI funding that established this research and to the Canola Council CARP funding for support to generate these unique genetic resources.

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8 Other Administrative Aspects

- **Establishment of a Canola Domestication Array**
  - This enables the rapid sequencing of key domestication genes that can be used to promote both the rapid survey of available germplasm generating details sequencing data for a subset of targeted genes. This information allows targeted selections in crossing schemes int be made.
- **Establishment of *B. oleracea* bridging lines that have returned to its diploid karyotype**
  - This enables development demonstrates that the removal of all aneuploid A-genome chromosomes in the *B. oleracea* background restores fertility in this diploid material. Currently, this material contains only one of the desired *B. napus* chromosomes. We now need to combine the two *B. napus* alleles on chromosome C6 and chromosome C7 necessary to restore fertility in synthetic tetraploid material.
- **Identification of additional germplasm from international germplasm collections**
  - Requests have been submitted and import paperwork obtained to facilitate the transfer of key Brassica germplasm that is currently lacking from the collection housed at the Plant Genetic Resources of Canada (PGRC). The request for the new germplasm has a focus on the C-genome (*B. oleracea*) where the deficit in genetic diversity occurs in both the *B. oleracea* collection the PGRC and perhaps more importantly in the *B. napus* germplasm collection. These germplasm will increase the content available to researchers and canola breeders in their efforts to adapt canala to future challenges.
- **Establishment of methodologies to generate CRISPR mutations in *Brassica oleracea***
  - This achievement enables targeted mutations to be generated in alleles of brassica species. The technology development is significant as it involves the use of tissue culture and regeneration. Now established, this opens to possibility to generate CRISPR mutations for other project goals.
- **Establishment of targeted CrispR mutation for selected *B. oleracea* genes**

– This material is necessary to interrogate the potential of the genes to reduce the hybridization barriers in hybridizations involving *B. oleracea* as a parent.

8.1 Project Personnel

Ms Kyla Horner - Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon. Responsible for:- Kyla is responsible for the generation of the A- and C-genome bridging lines. This work involves making the crosses, the embryo rescue, the tissue culture and plant recovery in soil. All plant surviving are genotyped to identify the present of the selected alleles and positive plants are backcrossed further. Kyla has also been responsible for the characterization and genotyping of the Brassica bridging species.

Mr Dallas Kessler - Plant Genetic Resources, Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon. Responsible for:- Dallas has been responsible for the organizing and receiving the seed requests from foreign collections. Dallas was responsible for the identification of material that was not present in the PGRC collection and augmented the collection in Canada. During this reporting period Mr Kessler retired after a fine career with AAFC.

9 Appendices

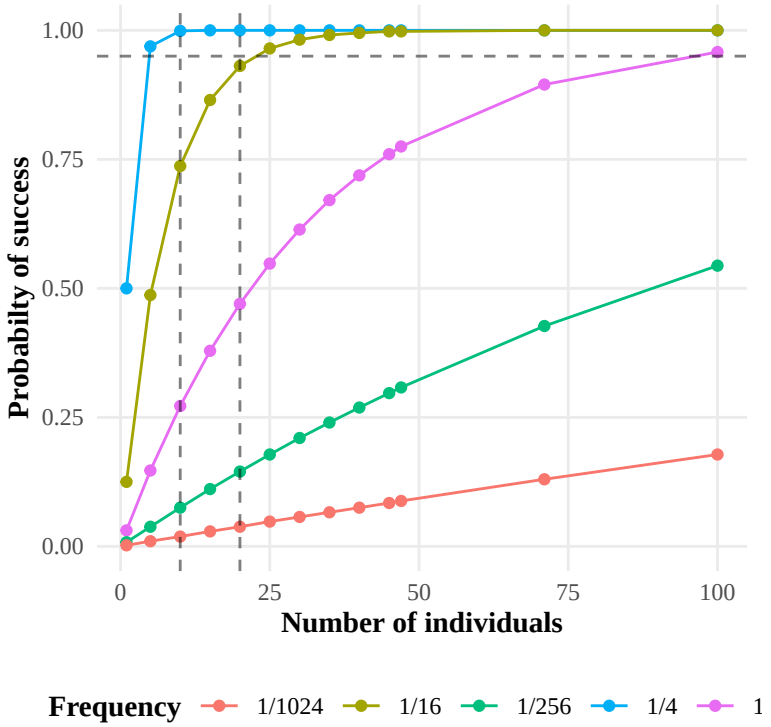


Figure 3: Graphical representation of the number of individuals required to be assessed to recover the desired genotype at different probabilty thresholds using a backcrossing strategy. The probabilty changes depending on the number of loci being selected. Probability curves are presented for one (yellow), two (pink), three (green), four (red) and five (blue) segreagting loci. The horizonal dashed line represents the 95% probabilty threshold. The vertical dashed lines indicate the probability values for ten and twenty individuals.

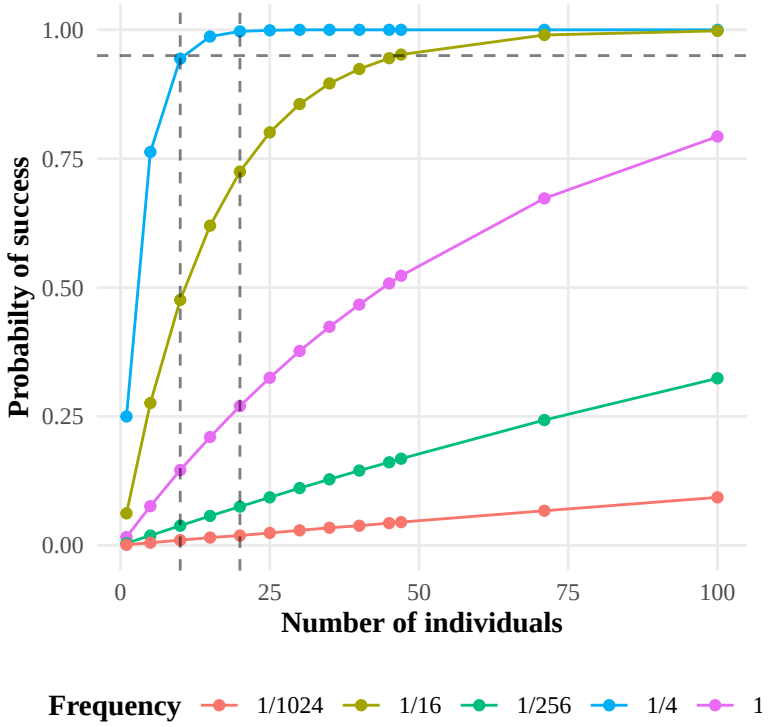


Figure 4: Graphical representation of the number of individuals required to be assessed to recover the desired genotype at different probabilty thresholds. The probabilty changes depending on the number of loci being selected. Probability curves are presented for one (blue), two (yellow), three (pink), four (green) and five (red) segreagting loci. The horizonal dashed line represents the 95% probabilty threshold. The vertical dashed lines indicate the probability values for ten and twenty individuals.

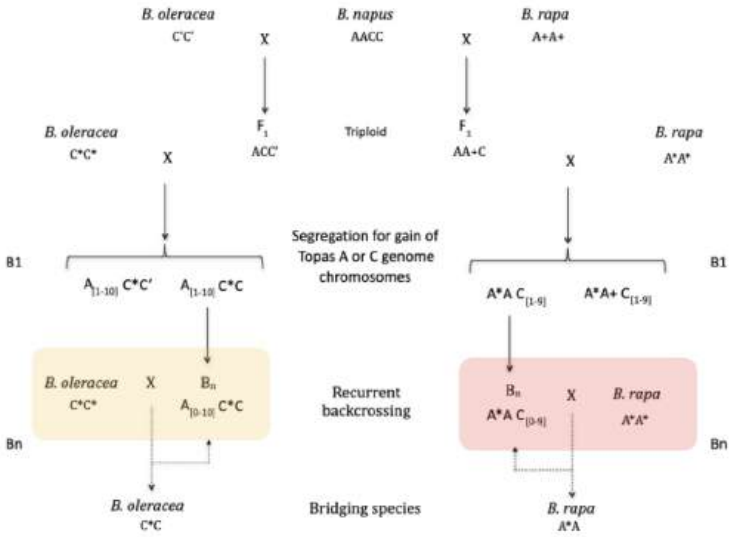


Figure 5: Restarted progress to generate the bridging lines required for crossing.

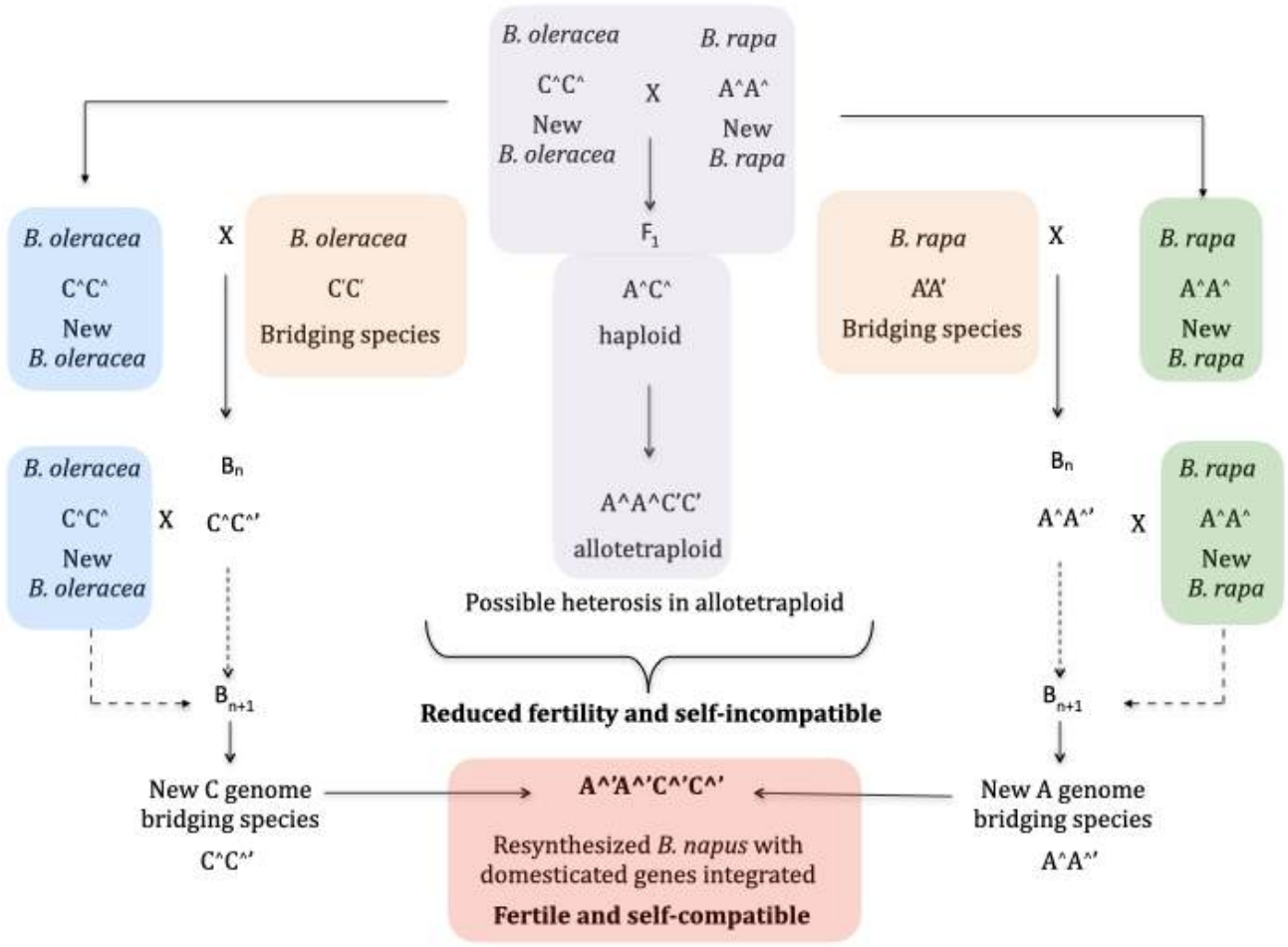


Figure 6: Summary of the strategy to generate new diversity in canola using bridging lines. Once the bridging lines are established, they will be crossed together to form newly synthetic *B. napus* gerplasm, only possessing the domesticated self-compatibility and recombination alleles from the canola variety Topas.

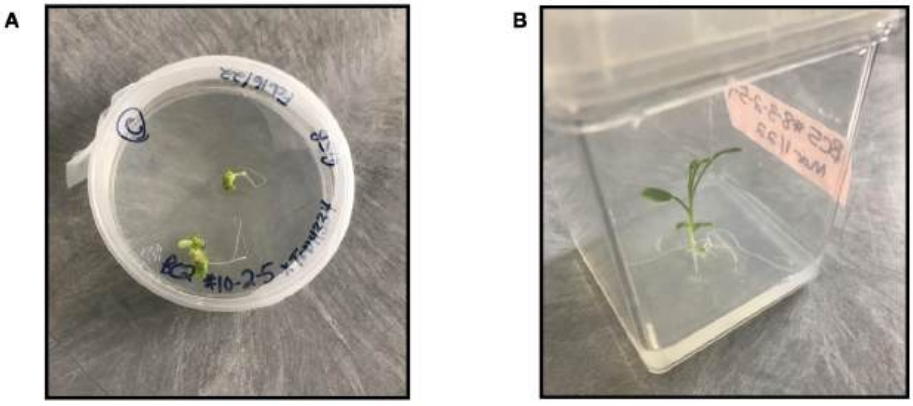


Figure 7: Examples of the material under development for the creation of the C-genome bridging lines. A) maintenance of material following embryo rescue and B) the development of roots in plantlet in tissue culture.

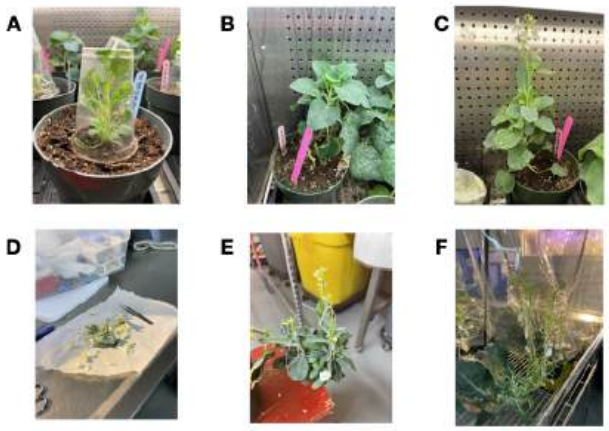


Figure 8: Example of the stages in the bridging species line development. A) Following the transfer of plants from tissue culture into soil, the material is maintained in a controlled enviroment and the humidity increased using simple plastic containers under the plant cutile is fully formed; B) The plants are allowed to grow and develop until they flower; C) when they are used in D) crosses to alternate genotypes; E) Buds are emmasuclated and the specific crosses labelled and pods are allowed to develop; F) Pods are opened and potential embryos are recovered and enter the tissue culture stage.

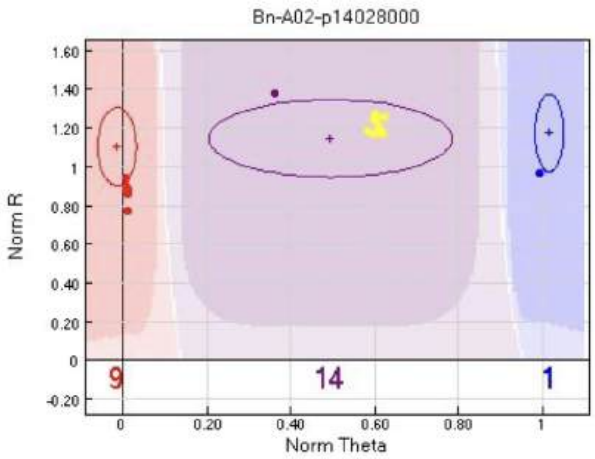
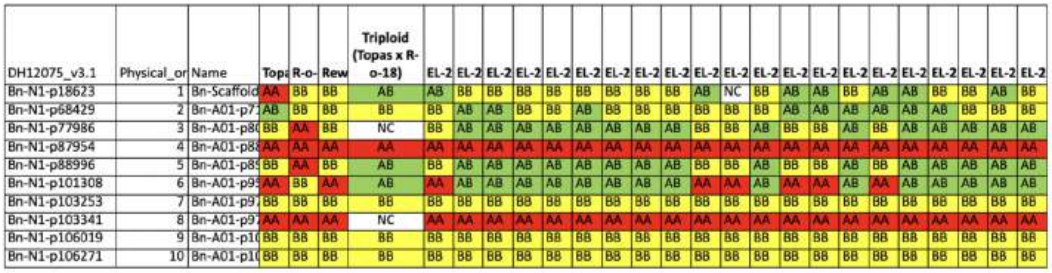


Figure 9: Example of the output from genotyping assays using the Illumina 90K SNP array. The individuals in blue represent homozgous alleles from the tetraploid *B. napus* genotype, the individuals in red possess homozygous alleles for the diploid *B. rapa* genotype. The individuals represented in purple possess alleles which are heterozygous containing both tetraplloid and diploid alleles. The data represents on locus from 90,000 loci throughout the genome and can be used to deterime the presence of aneuploud chromosomes.



DH12075_v3.1	Physical_or	Name	Topa	R-o-	Rew	Triploid (Topas x R-o-18)	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2	EL-2
Bn-N1-p18623	1	Bn-Scaffold	AA	BB	BB	AB	AB	BB	BB	BB	BB	BB	BB	BB	BB	AB	NC	BB	AB	AB	BB	AB	AB	BB	BB	AB	BB
Bn-N1-p68429	2	Bn-A01-p7	AB	BB	BB	BB	BB	AB	AB	BB	BB	AB	BB	BB	BB	BB	BB	BB	AB	AB	AB	AB	AB	AB	BB	BB	BB
Bn-N1-p77986	3	Bn-A01-p8	BB	AA	BB	NC	BB	AB	AB	AB	AB	AB	AB	AB	AB	BB	BB	AB	BB	BB	AB	BB	AB	AB	AB	AB	AB
Bn-N1-p87954	4	Bn-A01-p8	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
Bn-N1-p88996	5	Bn-A01-p8	BB	AA	BB	AB	BB	AB	AB	AB	AB	AB	AB	AB	AB	BB	BB	AB	BB	BB	AB	BB	AB	AB	AB	AB	AB
Bn-N1-p101308	6	Bn-A01-p9	AA	BB	AA	AB	AA	AB	AB	AB	AB	AB	AB	AB	AB	AA	AA	AB	AA	AA	AB	AA	AB	AB	AB	AB	AB
Bn-N1-p103253	7	Bn-A01-p9	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
Bn-N1-p103341	8	Bn-A01-p9	AA	AA	AA	NC	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
Bn-N1-p106019	9	Bn-A01-p1	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
Bn-N1-p106271	10	Bn-A01-p1	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB

Figure 10: Summary of the output from genotyping assays using Illumina SNP array. The first three columns describe the tetraploid SNP locus name, the physical order and its diploid genome name. The individuals are represented in the remaining columns with tetraploid genotype (Topas), the diploids genotypes (R-o-18 & Reward) and the triploid genotype (Topas x R-o-18) indicated for each locus. The backcross individals labelled EL-### and their genotypes are presented. The genotypes are colured to indicate the presence of homozygous or heterozygous alleles. selection of the optimal genotypes can be made from the availble material.

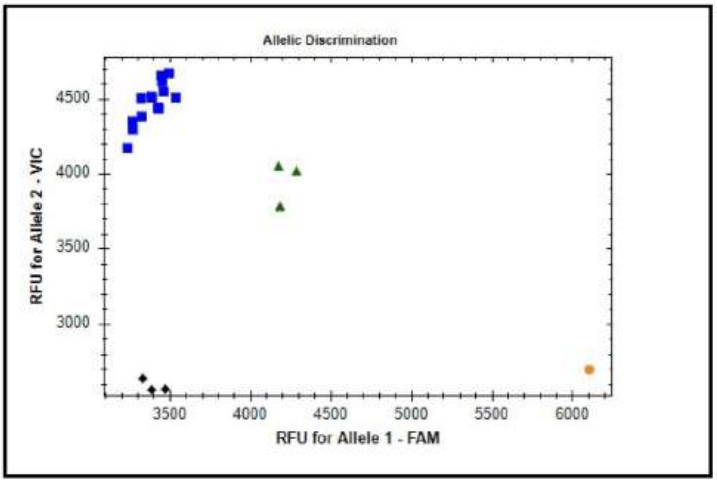


Figure 11: Example of the output from genotyping assays using the newly developed KaspR marker technology. The individuals in blue represent homozgous alleles from genotype 1, the individuals in black possess homozygous alleles for genotype 2. The individuals represented in green possess alleles which are heterozygous. These data can be rapid obtained and used for clear diagnostic decisions.

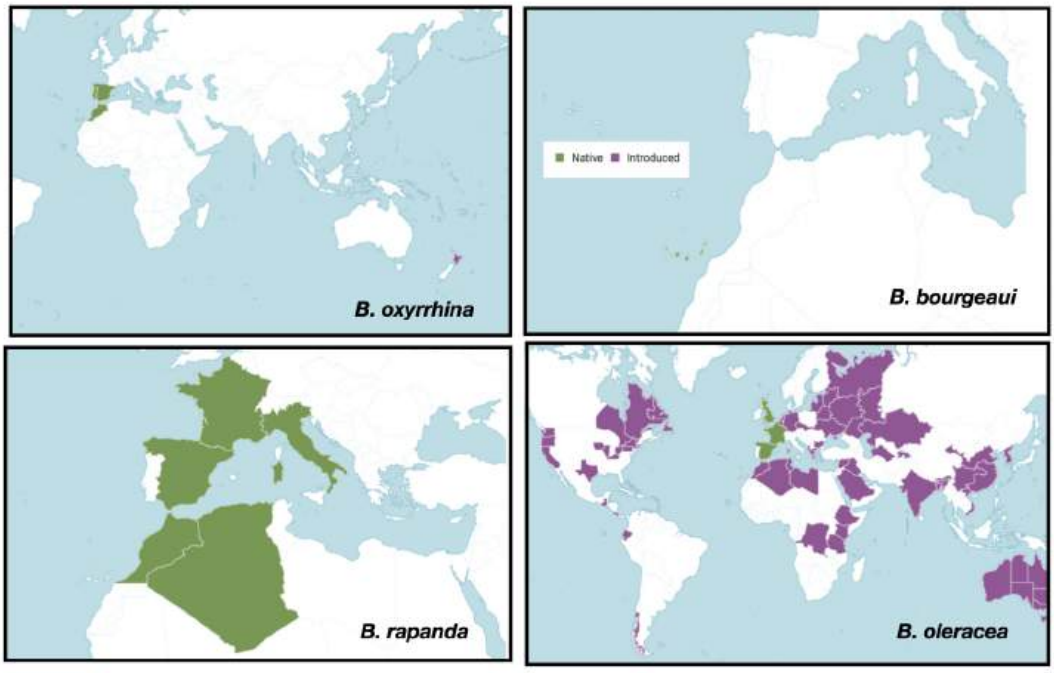


Figure 12: Graphical representation of the native range of four species selected from the Spanish genebank. Those regions in green represent he native range of the species and the regions in purple represent areas into which the species have been introduced.

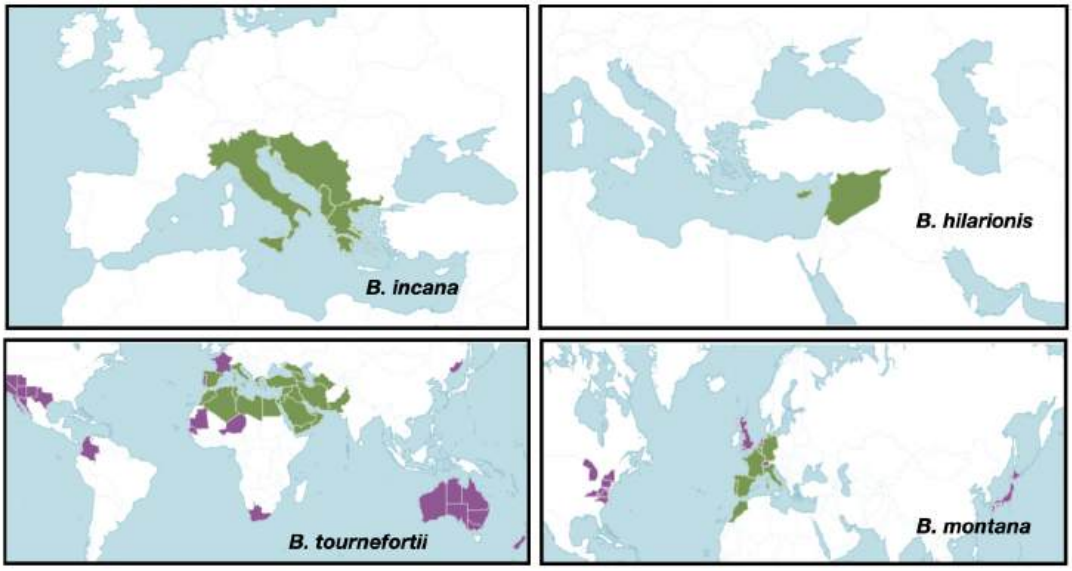


Figure 13: Graphical representation of the native range of four species selected from the C-genome genebank collection. Those regions in green represent he native range of the species and the regions in purple represent areas into which the species have been introduced.

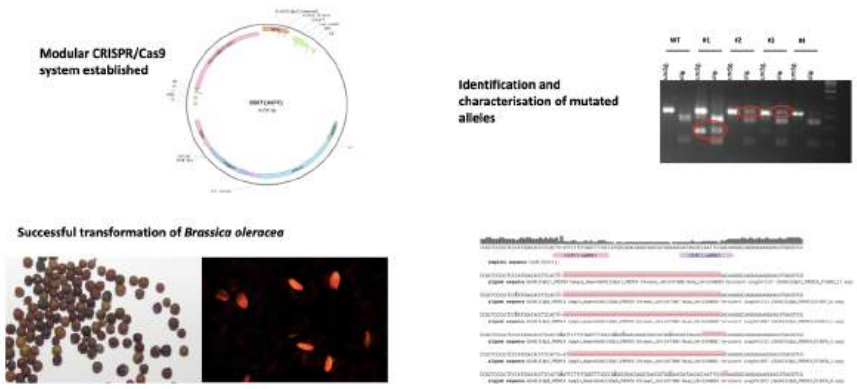


Figure 14: A summary of the CRISPR process. A) the modular vector system used to generate CRISPR mutations; B) Idnetifection of mutated alleles in lines expressing the CAS9 and guide RNA; C) The deisgn of guide RNA sequences to generated targted mutations; D) The identification of transgenic *B. oleracea* lines using a fluorescent marker.

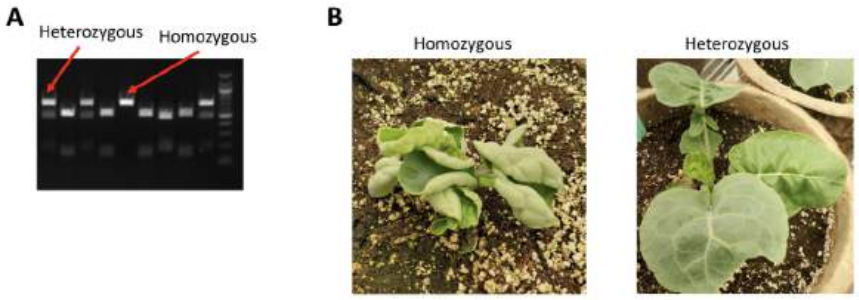


Figure 15: Production of a DRD1 mutant line. A) RFLP genotyping result of the segregating drd1 mutant allele. Mutant alleles are resistant to restriction enzyme digestion resulting in an undigested PCR band on agarose gel. B) Representative picture of homozygous and heterozygous plants for the drd1 allele. The homozygous mutant reproducibly shows an upward curling of true leaves.