SaskCanola/SaskWheat Project Final Report

1. **Project title and reporting period.**

   Title: Manipulating Recombination in Polyploid Crops
   Reporting period: April 1st 2017 – March 31st 2024.

2. **Name of the Principal Investigator and contact information.**

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3. **Name of the collaborators and contact information**

   Collaborators: Sateesh Kagale (National Research Council Canada), Curtis Pozniak (University of Saskatchewan), Sue Armstrong (University of Birmingham, UK).

4. **Abstract.** *Describe in lay language the progress towards the project objectives over the last reporting period. Include any key findings and any interim conclusions. Include any deviations from the original methodology.*

   Accelerating crop breeding to improve productivity is a global priority. One of the key limitations in this goal is the reduction in genetic diversity due to continuous selection for a small number of traits in standard breeding programs. The ability to make improvements to crops relies on our ability to introduce novel genetic variation, which is dependent upon natural levels of chromosome pairing and recombination during meiosis. This project carried out research to provide novel information on the role of a number of candidate meiotic genes in chromosome pairing and recombination, potentially offering a mechanism to influence chromosome pairing in canola and wheat, which could significantly impact future crop improvement. The primary objective of the project was to create novel variation by manipulating genes impacting homoeologous recombination (chromosomal exchange between the constituent genomes) in polyploid crops; exploiting CRISPR/Cas9 technology to generate gene knockouts.

   - During the course of the project further work was completed to hone methods for analysing homoeologous recombination in *Brassica napus*. This resolved the regions of the genome controlling normal homologous recombination leading to a publication in New Phytologist (Higgins et al, 2021). This publication was highlighted by an accompanying commentary written by two leading experts in plant meiosis. (Sourdille and Jenczewski, 2021).

   - Analyses of the gene expression data (RNASeq data) comparing meiosis in a stable and unstable *B. napus* line proved very informative; confirming the likely candidate gene controlling homoeologous pairing, and identifying additional genes which could prove valuable in manipulating meiosis in plants. A manuscript has been submitted for peer review.

   - Transformation of both canola and wheat with gRNAs targeting a number of meiosis specific genes (six in canola and three in wheat) was successful and a number of different events were identified. Although analyses of these lines is still ongoing varying phenotypic effects related to fertility were identified, suggesting the efficacy of the mutations and providing information on the role of each candidate gene.

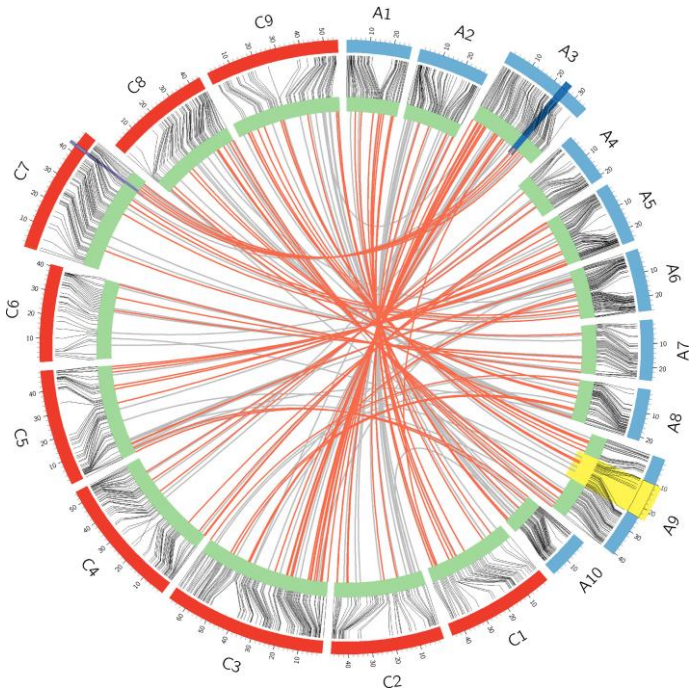
5. **Introduction:** *Brief project background and rationale.*

The ability to make further crop improvements relies on the introduction of novel allelic variation, one such source being related species; however, interspecific barriers to recombination can limit our ability to transfer new variation into the crops. In addition, there is evidence that breeding has inadvertently selected for a number of naturally occurring inter-specific genome recombination events within the polyploid nucleus, which have provided an advantage either to yield (Quijada et al, 2006) or quality traits (Chalhoub et al, 2014). Thus the ability to increase recombination between the constituent genomes of the polyploid crops could lead to changes in gene copy number and associated selectable variation that maybe beneficial.

Most polyploid species appear to have evolved mechanisms to prevent homoeologous chromosome pairing between their progenitor genomes in order to ensure fertility in subsequent generations. Both wheat and canola have such mechanisms. In wheat the locus *Ph1* (pairing homoeologous 1) was identified in deletion lines that showed high levels of aberrant pairing between the three progenitor genomes and showed enhanced crossovers in interspecific hybrids (Moore, 2014); only recently the molecular identity of the gene(s) controlling this process has been proposed. In wheat recent evidence points to novel tandem duplicate of *ZIP4*, a homologue of yeast *Spo22*, as the major gene controlling homoeologous chromosome pairing (Abdullah et al, 2021), but this is unlikely to be the case in *Brassica*. The phenotype for *Ph1* in wheat is extreme and is only seen in deletion lines, no natural variation has been found, unlike *B. napus* which showed a low but measurable amount of HeR even in elite lines (Higgins *et al*., 2018). In canola, work completed during the course of the project further resolved one very strong locus and two minor loci (**Figure 1**) controlling pairing between homoeologues (Higgins et al, 2021). More recent work in the current project has further implicated two particular candidates as targets for manipulating recombination in polyploids.

The project aimed to manipulate candidate genes underlying the major locus identified in canola and to target loci in wheat based on our cumulative knowledge to assess their ability to control homologous pairing and their potential to facilitate interspecific exchanges. This project aimed to provide novel information on the role of these candidate genes in chromosome pairing and recombination, potentially offering a mechanism to influence homologous and homoeologous pairing in polyploid crops, which could significantly impact future crop improvement.

Figure 1: Map positions of QTL controlling homoeologous pairing events in *Brassica napus*. The outer circle represents the physical length of the chromosomes (A genome in blue, C genome in red), the inner circle (green) the genetic linkage groups, the position of the markers on the physical chromosomes is shown by the linked grey lines. The positions of the QTL loci are shown by coloured blocks, with the colours representing the different phenotypes used to identify loci; purple – HeR only, blue – HeR and cytogenetics, and yellow – common to all phenotypes. The synteny between *B. napus* meiosis genes are shown as connecting lines across the centre of the circle, those genes with only two orthologues are shown in red (*Published in Higgins et al, 2021*).



6. Objectives and the *progress towards meeting each objective*

Objectives (*Please list the original objectives and/or revised objectives if Ministry-approved revisions have been made to original objective. A justification is needed for any deviation from original objectives)*	**Progress (e.g. completed/in progress)**
1. Identify homologues of gene candidates controlling homoeologous recombination in wheat and *Camelina sativa*.	**Complete.** Homologues of the gene candidates have been identified in wheat and camelina for the initial targets; but additional genes have been targeted in canola due to the success of the protocol.
2. Develop constructs for gene knock-outs using CRISPR technology	**Complete:** Constructs have been generated to target multiple genes in both canola and camelina. Tranformation has been completed for all current *B. napus* targets. Wheat constructs have been generated and transformed successfully into bread wheat.
3. Assess levels of homoeologous recombination in crops	**On-going.** Various methods are being used to assess the levels of both homologous and homoeologous recombination in the edited lines. *Brassica napus* transgenic lines have been crossed to natural *B. napus* lines to assess recombination by studying the impact of the mutant alleles on fertility and recombination in the resultant progeny. Cytological work and plant fertility are also being used as tools to assess the impact of the gene editing on recombination.

Please add additional lines as required.

7. Results and discussion: *Describe research accomplishments during the reporting period under relevant objectives listed under section 6. The results need to be accompanied with tables, graphs and/or other illustrations. Provide discussion necessary to the full understanding of the results. Where applicable, results*

should be discussed in the context of existing knowledge and relevant literature. Detail any major concerns or project setbacks.

Objective 1: Identify candidate genes in multiple polyploid species.

Although polyploid species are likely to have independently evolved mechanisms to control such aberrant pairing it would be expected, due to the fundamental nature of meiosis and recombination and the high degree of conservation of the corresponding biochemical and cell biology processes across sexually reproducing organisms, that genes that impact homoeologous pairing in one species would have similar effects in other polyploids.

Although our colleagues in the UK had provided some meiocyte data for *B. napus* it was somewhat limiting, without sufficient replication to confirm observed expression patterns. In order to provide confirmatory data for candidate genes and to facilitate downstream analyses of mutated lines protocols were established for extracting meiocyte tissue from *B. napus*. Our colleague, Dr. Kagale, had developed protocols for such work in wheat and assisted in adapting those protocols for *B. napus*. The size of the chromosomes in *B. napus*, at least fourteen times smaller than wheat, makes staging the different events in meiosis difficult; however, we have been able to reliably extract meiocytes during prophase when recombination should be taking place. This method has been used to extract meiocytes from an established canola cultivar and a newly resynthesized line, which was confirmed to be undergoing homoeologous recombination. This technique was applied to one of our mutant lines developed in Objective 2.

RNASeq analyses was performed on replicated samples from multiple stages of meiosis in two lines that vary significantly for their ability to control the level of homoeologous pairing. A newly hired bioinformatician at AAFC who has extensive experience of RNASeq analyses in polyloid species, Dr. Peng Gao, assisted with analyses of these data for publication (**Figure 2** and **Figure 3**). This will be the first such analyses published for *B. napus* and as such is expected to be quite impactful, the manuscript describing the data has been submitted for peer review. A number of notable differences in gene expression of meiotic genes was found between the natural *B. napus* line and the resynthesized line, which mostly related to either the level of expression or the timing of gene expression (**Figure 3**). In order to assist with identifying the likely candidate gene for the *BnPh1* QTL on A09 the expression of all genes underlying the QTL region were assessed with a focus on known meiotic genes (**Figure 4**), the homologue of *Mus81* was the only gene that showed differential expression between the two lines. Mus81 has been reported to participate in DNA repair; however, its exact function still remains unclear. Thus work completed during this project will be very informative in resolving this question. Interestingly a recent publication has suggested that the protein may play a crucial role in resolving atypical meiotic intermediates (Mu et al, 2023) which may be relevant to its role in *B. napus*. The RNASeq analyses identified a number of additional genes which could contribute to the minor QTL loci and would warrant further study.

Figure 2: Principal Component Analyses (PCA) of expression data in the six stages of meiosis of *B. napus;* in two lines differing significantly in their ability to control normal chromosome pairing (DH12075-stable; RB1-4-unstable). The PCA clearly differentiates three phases of meiosis and fundamental differences between the two *B. napus* lines.

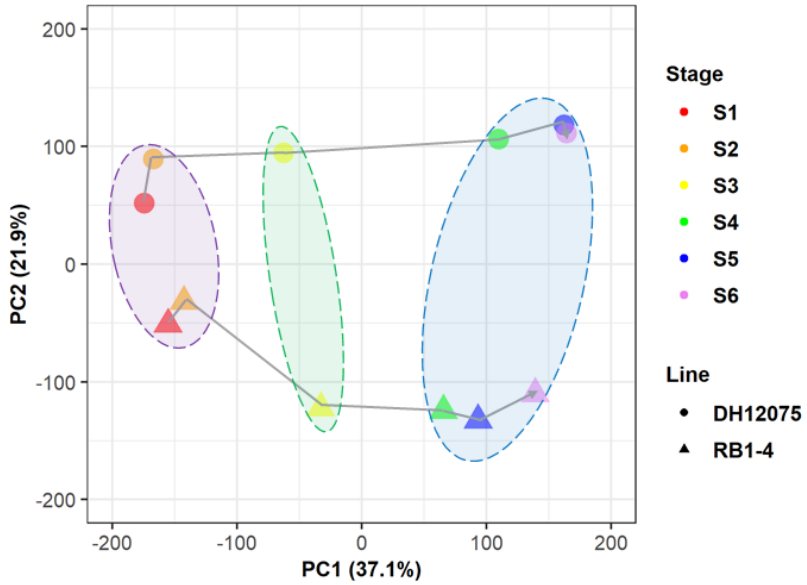


Figure 3: Hierachical clustering of all known meiotic genes during six phase of meiosis (Prophase I and Metaphase I). Three clusters were clear, Cluster 1 members (124/155, 80.0%) were highly expressed in DH12075 with average 1.83 times higher (normalized counts) expression than in RB. Cluster 2 members (50/58, 86.2%) highly expressed in RB with average 2.84 times higher (normalized counts) than in DH. Cluster 3 highly expressed in early stages. Totally, 95.6% (151/158) genes in DH and 98.7%(156/158) genes in RB have higher expressions in early stages (S1 to S3) than late stages (S4 to S6).

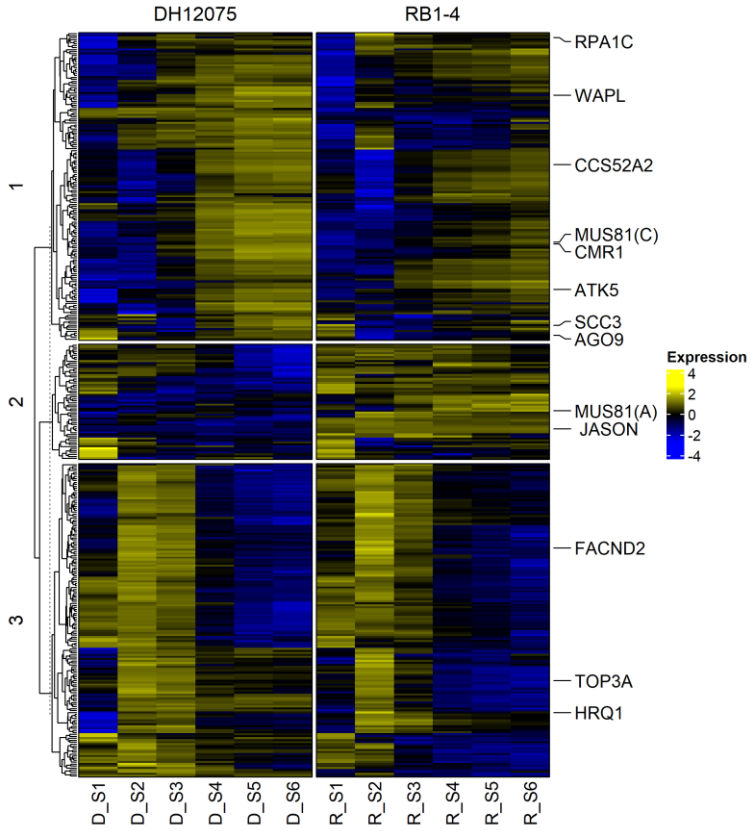
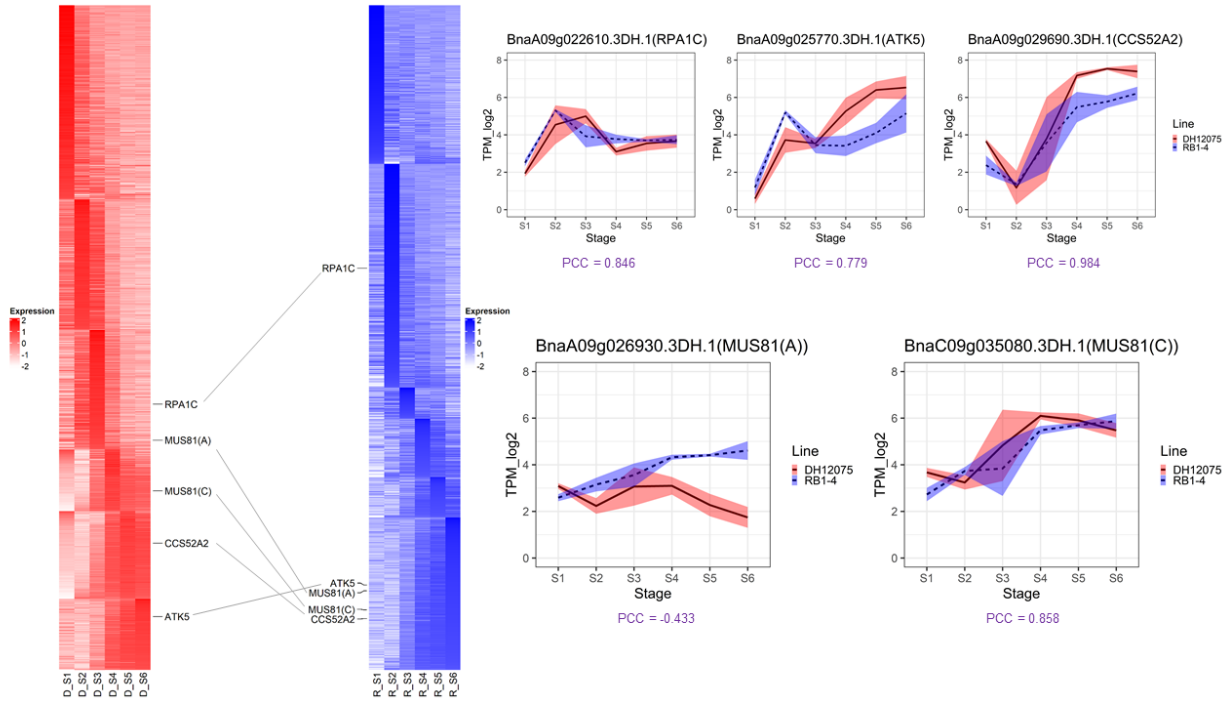
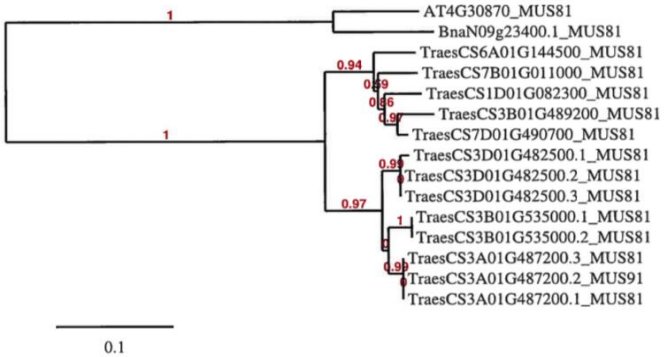


Figure 4: Differential gene expression analyses during *B. napus* meiosis. All differentially expressed genes as identified in DH12075 (in red) and RB1-4 (in blue) are shown in the heatmap to the left. On the right the expression pattern of candidate genes underlying the QTL controlling homoeologous recombination in *B. napus* are shown. The homologue of MUS81 on A9 (QTL region) is showing clear differences in expression pattern between the stable and unstable *B. napus* in comparison with the orthologous copy on C9.



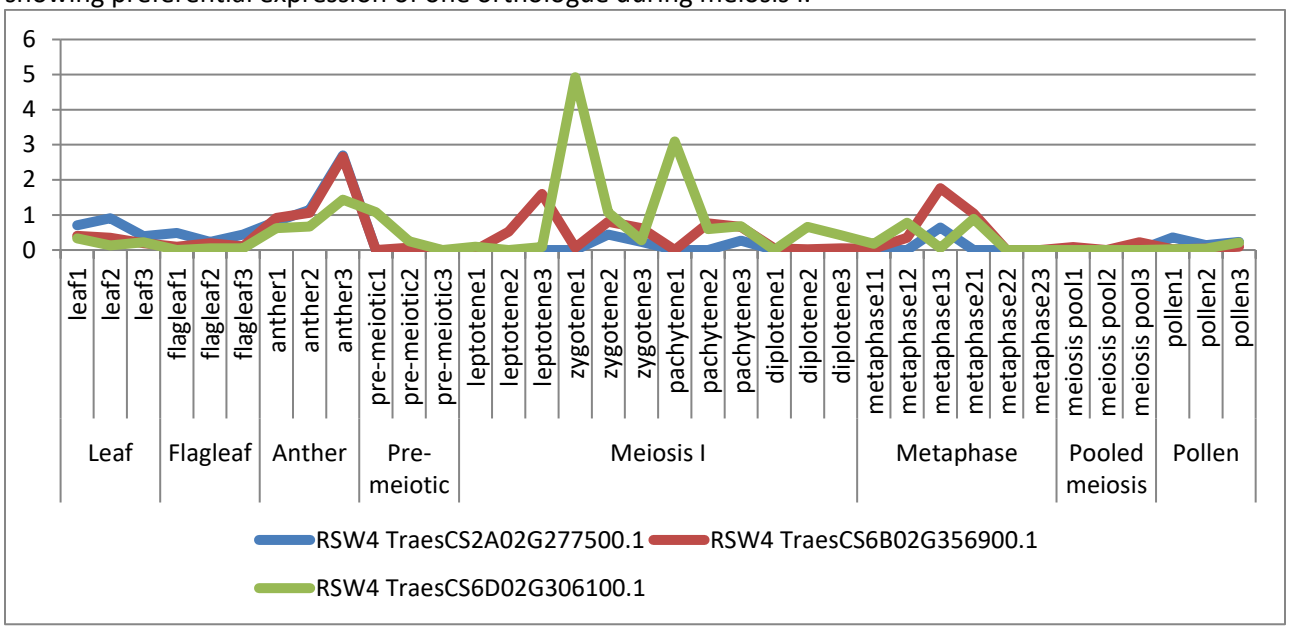
Dr. Kagale provided pre-publication access to extensive transcriptome data from wheat meiocyte tissue at all stages of meiosis. Initially all wheat orthologues of four candidate recombination genes were identified through sequence homology (**Figure 5**).

Figure 5: Example phylogenetic tree for wheat orthologues of candidate gene.



The expression data was extracted for each of the orthologues and compared across the different stages of meiosis to identify those genes being preferentially expressed in prophase while being specific to meiotic tissue, that is not expressed in leaf tissue. The number of orthologous genes ranged between three and 15 copies within the wheat genome, but in each instance either one or two homologous genes appear to be preferentially expressed during the expected stages (**Figure 6**). Appropriate guide RNAs (gRNA) that target these particular orthologues are being designed using the WheatCRISPR tool developed by Dr. Kagale.

Figure 6: Replicated gene expression data across multiple stages of meiosis for one candidate gene, showing preferential expression of one orthologue during meiosis I.



Objective 2: Development of CRISPR constructs for gene inactivation.

CRISPR/Cas9 technology has been widely adopted as a tool for gene manipulation due to the relative ease of application. The technology relies upon specific binding between a gRNA and the target gene sequence, which then directs binding of Cas9 that generates a double-strand break (DSB), the resulting error prone repair of these DSBs can create a range of mutations which disable the target gene.

To enable CRISPR-mediated inactivation of candidate genes, at least two specific guide RNAs were designed for each target gene in *B. napus*. All *B. napus* transformations were successfully completed, including constructs for two additional targets, one which should function as a positive control and one as a negative. All the transformants, some of which are now at the T3 generation, have been tissue sampled, DNA extracted and the candidate locus sequenced to assess the presence of mutations (**Table 1**). This work has been very effective, mostly multiple independent mutations have been identified per target gene. In one instance (ZIP4) the mutation rate was unexpectedly high at 51% (compared to range of 3-6%) identified for a single locus, either suggesting the gRNA was extremely efficient or that the target itself may be influencing the impact of the Cas9 editing. A summary of all the available gene edited plants is provided in Table 1. Some of the edited lines, in particular where both homologues of a gene have been affected in the same line, have required additional cloning of the target site, rather than direct amplification and resequencing of the site, which took longer than anticipated.

Table 1: Summary of all available gene edited lines in *Brassica napus*.

B. napus gene	Common Name	Current Generation	Single or Multiple Edits	Homozygous
BnaN09g30610	MEI2-like protein	T3	Single	YES
BnaN06g31610	ZIP4	T2	Single	YES
BnaN17g33570	ZIP4	T2	Multiple	YES
BnaN03g48460	MSH3	T2	Multiple	YES
BnaN17g47090	MSH3	T2	Multiple	NO*
BnaN09g23400	MUS81	T3	Multiple	YES
BnaN17g39170	RAD18	T2	Single	YES
BnaN09g19560	RPA70C	T2	Multiple	YES

*There is one set of lines carrying homozygous edits for both copies of MSH3 (N03 and N17); one set of lines homozygous for an edit in just N03 and additional lines which are heterozygous for edits in N17. Interestingly the three sets of lines show an additive effect on the phenotype, with an increased impact on fertility when two of the copies are mutated. In addition, knocking out one of the two copies shows a minimal impact, suggesting genome bias.

In wheat, advised by Dr. Kagale between one and two gRNAs were designed for the candidate genes and constructs were provided by AAFC to NRC. Wheat transformation is more difficult and expensive than *B. napus* transformation, thus perhaps extra care was taken in selection of gRNA, choice of vector, and design of construct. Although this took some time, four constructs that target three candidates were developed for wheat, each was sequenced to confirm the identity of the cloned gRNA and the vectors transformed into an agrobacterium strain. However, after initial unsuccessful agrobacterium mediated transformation wheat transformation, the constructs were remade to allow biolistic transformation which proved successfully for all 4 constructs. A total of 48 transformed lines were obtained, of which 41 plants tested positive for BAR gene, indicating successful plant transformation in 85% of the individuals (**Table 2**). The target genes were amplified by PCR and sequenced using Sanger sequencing. Four out of the seven transformants obtained for MSH3-gRNA1 construct showed either a single bp deletion or substitution (A->T or G->T) at the target site (ie., three bp upstream of the PAM site). Only one of out the 10 transformed lines obtained for SYN4-gRNA1 showed a single bp substitution (G->A) at the target site. None of the primary transformants for both constructs designed to target RSW4 genes showed any edits.

Table 2: Summary of wheat transformation

Construct	Number of plants	+ve PCR test for bar gene	no bar gene
MSHS-gRNA1	7	7	0
RSW4-gRNA1	16	13	3
RSW4-gRNA2	14	11	3
SYN4-gRNA1	11	10	1
Total	48	41	7

A total of 261 plants from M_0 were advanced to M_1 generation. DNA was isolated from all 261 lines, the target genes were amplified by PCR and sequenced using Sanger sequencing. A total of 33 lines (16 for MSH3-gRNA1, 12 for Syn4-gRNA1 and 5 for RSW4-gRNA1) showed edits (deletions or substitutions) at

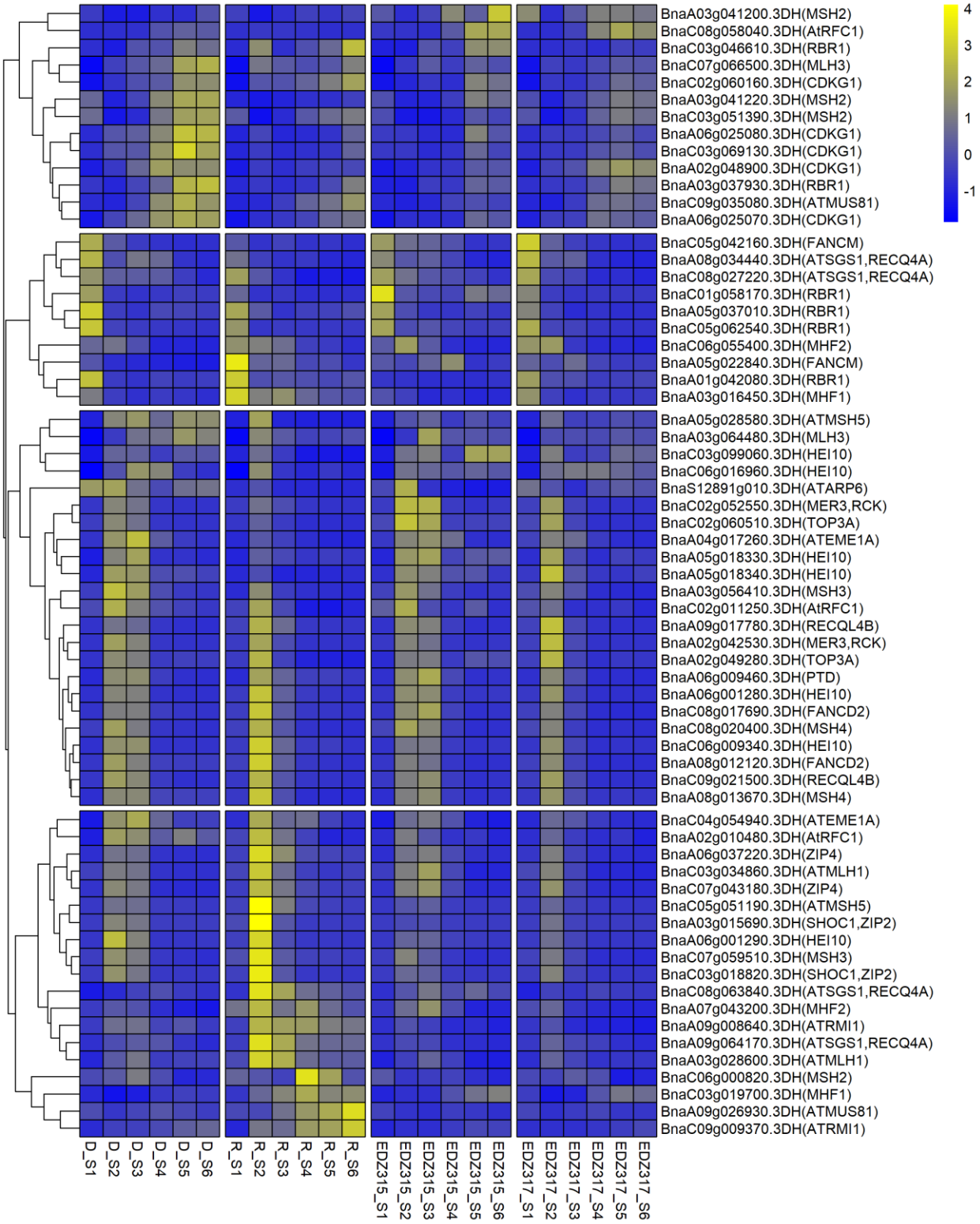
the target site. Several phenotypes consistent with meiotic mutants, including dwarfism, reduced tillering, late flowering or maturity, and/or production of very few seeds, were observed in the mutant lines. Some of the edited lines also did not survive, which is also consistent with phenotypes of defective meiotic genes in other crops.

Objective 3: Characterisation of candidate gene function.

Characterisation of B. napus *gene edited lines.*
Established methods for assessing HE in *B. napus* are being applied to the *B. napus* gene-knockout lines (Higgins et al, 2018, G3, 8: 2673-2683), but we are looking to additional methods which might allow us to increase our ability to study the trait. Studying meiosis is notoriously difficult and thus requires multiple approaches, especially in a species with small chromosomes.

All the meiosis-specific CRISPR mutant lines for *B. napus* have been characterized at the molecular level in the T2 generation. They have in the most part been shown to be homozygous for the target gene with no off-target effects (**Table 2**). Physiological data pertaining to morphology and flowering time has been collected for each of the mutant T2 lines, test crosses have been carried out for the lines as they become available. Several T2 lines have been identified with severe morphological and altered flowering time phenotypes, in particular the lines carrying mutations in the key candidate genes, these lines are now the focus of additional analyses. Due to the difficulties of accurately assessing recombination in the edited lines protocols are being developed for direct measurement of recombination in, which could become an invaluable tool for detecting *de novo* chromosomal rearrangements in *Brassica napus*. However, this work is still ongoing and will continue beyond the end of the project. In addition, cytogenetic methods are being used to analyze the meiotic chromosomes in the stable and unstable *Brassica napus* genotypes, and putative CRISPR meiosis-specific lines. Because of the obvious difference in gene expression for Mus81 between the natural and resynthesized *B. napus* line, much of our work has focused on studying the edited lines for this genes. We are still collecting recombination data from the edited lines which will be reported on after the completion of the project. Staged meiocyte tissue was collected from two gene edited BnA09 *Mus81* CRISPR lines and a time-coursed RNAseq analysis was completed similar to that described in Objective 1, since this proved very informative for our understanding of meiosis in *B. napus*. These data have been analysed and a manuscript describing these analyses is being drafted for submission. The impact of the gene editing seemed to be very specific significantly impacting the expression of a small number of genes, which changing the timing of expression of sets of key meiotic genes (**Figure 7**).

Figure 7: Hierarchical clustering heat map for meiosis genes in two CRISPR lines with independent edits of BnMus81. The panels from left to right show the two control lines, DH12075 and RB1 and then the two edited lines. BnMus81 is shown in the bottom cluster with no expression in the edited lines. The edited lines both show a change in timing of gene expression for a number of key crossover genes, with lower expression in stage 3, which is a similar pattern to the resynthesisied lines.


BnaA03g041200.3DH(MSH2)
BnaC08g058040.3DH(AtRFC1)
BnaC03g046610.3DH(RBR1)
BnaC07g066500.3DH(MLH3)
BnaC02g060160.3DH(CDKG1)
BnaA03g041220.3DH(MSH2)
BnaC03g051390.3DH(MSH2)
BnaA06g025080.3DH(CDKG1)
BnaC03g069130.3DH(CDKG1)
BnaA02g048900.3DH(CDKG1)
BnaA03g037930.3DH(RBR1)
BnaC09g035080.3DH(ATMUS81)
BnaA06g025070.3DH(CDKG1)
BnaC05g042160.3DH(FANCM)
BnaA08g034440.3DH(ATSGS1,RECQ4A)
BnaC08g027220.3DH(ATSGS1,RECQ4A)
BnaC01g058170.3DH(RBR1)
BnaA05g037010.3DH(RBR1)
BnaC05g062540.3DH(RBR1)
BnaC06g055400.3DH(MHF2)
BnaA05g022840.3DH(FANCM)
BnaA01g042080.3DH(RBR1)
BnaA03g016450.3DH(MHF1)
BnaA05g028580.3DH(ATMSH5)
BnaA03g064480.3DH(MLH3)
BnaC03g099060.3DH(HEI10)
BnaC06g016960.3DH(HEI10)
BnaS12891g010.3DH(ATARP6)
BnaC02g052550.3DH(MER3,RCK)
BnaC02g060510.3DH(TOP3A)
BnaA04g017260.3DH(ATEME1A)
BnaA05g018330.3DH(HEI10)
BnaA05g018340.3DH(HEI10)
BnaA03g056410.3DH(MSH3)
BnaC02g011250.3DH(AtRFC1)
BnaA09g017780.3DH(RECQL4B)
BnaA02g042530.3DH(MER3,RCK)
BnaA02g049280.3DH(TOP3A)
BnaA06g009460.3DH(PTD)
BnaA06g001280.3DH(HEI10)
BnaC08g017690.3DH(FANCD2)
BnaC08g020400.3DH(MSH4)
BnaC06g009340.3DH(HEI10)
BnaA08g012120.3DH(FANCD2)
BnaC09g021500.3DH(RECQL4B)
BnaA08g013670.3DH(MSH4)
BnaC04g054940.3DH(ATEME1A)
BnaA02g010480.3DH(AtRFC1)
BnaA06g037220.3DH(ZIP4)
BnaC03g034860.3DH(ATMLH1)
BnaC07g043180.3DH(ZIP4)
BnaC05g051190.3DH(ATMSH5)
BnaA03g015690.3DH(SHOC1,ZIP2)
BnaA06g001290.3DH(HEI10)
BnaC07g059510.3DH(MSH3)
BnaC03g018820.3DH(SHOC1,ZIP2)
BnaC08g063840.3DH(ATSGS1,RECQ4A)
BnaA07g043200.3DH(MHF2)
BnaA09g008640.3DH(ATRMI1)
BnaA09g064170.3DH(ATSGS1,RECQ4A)
BnaA03g028600.3DH(ATMLH1)
BnaC06g000820.3DH(MSH2)
BnaC03g019700.3DH(MHF1)
BnaA09g026930.3DH(ATMUS81)
BnaC09g009370.3DH(ATRMI1)
4
3
2
1
0
-1
D_S1
D_S2
D_S3
D_S4
D_S5
D_S6
R_S1
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Crosses have been made between the edited lines and an established B. napus line in order to assess recombination levels but these analyses are ongoing. Analyses of the other edited lines is also in progress.

Characterisation of wheat gene edited lines

The gene editing efficiency in polyploid wheat is notably low. Consequently, to achieve modifications in all three homoeologs of a gene, edited plants must be progressed, and in each successive generation, mutants must undergo sequencing to identify lines harboring homozygous mutations in all three homoeologs. As a result, a total of 178 M_1 plants were advanced to produce M_2 seeds. Among these, 8 independent mutants (3 for MSH2, 3 for RSW4, and 2 for SYN4) were selected due to their edited status and concurrent display of reduced fertility, which is a phenotypic manifestation of meiotic defects and chromosome pairing issues. Subsequently, 10 seeds from each of these 8 mutant plants were further developed into M_3, with mutations confirmed via Sanger sequencing, and these plants underwent comprehensive phenotyping for pollen viability and chromosome pairing behavior. **Figures 8, 9, and 10** illustrate photographic comparisons between the three mutants and wildtype fielder plants.

The pollen viability among MSH3 M_3 mutants varied widely, ranging from 0 to 98%, with total seed production per plant spanning from 0 to 195 seeds (**Figure 11**; **Table 3**). RSW4 M3 mutants displayed pollen viability ranging from 79 to 98%, with total seed production ranging from 23 to 181 seeds per plant. SYN4 M_3 mutants exhibited pollen viability ranging from 96 to 98%, with total seed production ranging from 42 to 131 seeds per plant. In contrast, wildtype Fielder plants consistently produced between 130 and 180 seeds per plant, with pollen viability exceeding 90%. These findings indicated that MSH3 mutants exerted the most significant impact on pollen viability and seed set, followed by RSW4 mutants. SYN4 mutants demonstrated nearly identical fertility levels to wildtype Fielder plants, suggesting that the SYN4 mutation had negligible effects on wheat fertility.

Figure 8: MS3H3 M3 mutant. Photographs comparing the MSH3 mutant (on right) with that of the wildtype Fielder plant (on left). The mutant lines displayed shorter stature, delayed flowering, and reduced seed production compared to the wild type. Certain plants failed to yield seeds, while others produced defective chaffy seeds. Moreover, there were observable differences in the spike morphology compared to the wild type plants.

Figure 9: RSW4 M3 mutant. Photograph comparing the mutant line (on right) with wild type Fielder (on left). The mutant lines also displayed delayed flowering, and reduced seed production, compared to the wild type but this was less pronounced than for the MSH4 mutants.

Figure 10: SYN4 M3 mutant. Photograph comparing the mutant (on right) with wild type Fielder (on left). The mutant lines displayed dwarf stature and were also delayed in flowering, but seed production was not affected.

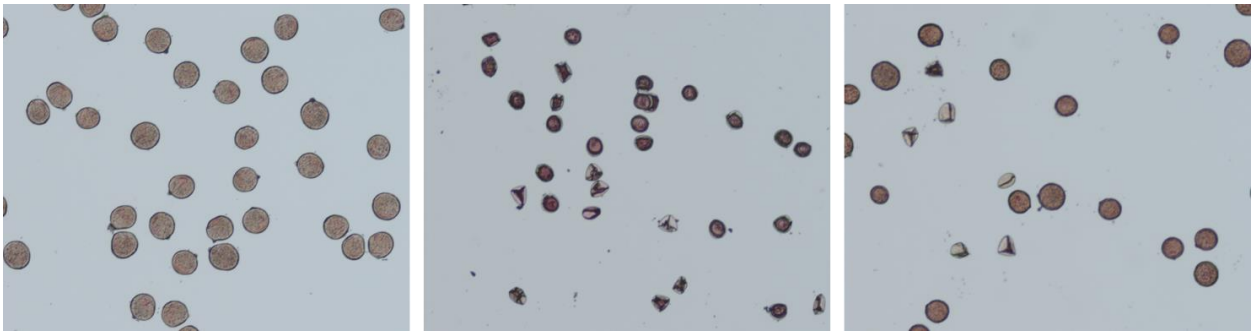
Table 3. Pollen viability and seed set in M_3 mutants

Plant name	Construct	Spikes No	Total No. of seeds	Inviable pollen grains (%)				Pollen viability (%)
				Rep1	Rep2	Rep3	Average	
8165	MSH3-gRNA1	10	52	16.04	13.58	17.33	15.65	84.35
8165	MSH3-gRNA1	8	85	8.43	12.82	7.91	9.72	90.28
8165	MSH3-gRNA1	9	67	10.34	7.89	11.48	9.90	90.10
8165	MSH3-gRNA1	6	195	1.02	1.14	1.12	1.09	98.91
8165	MSH3-gRNA1	11	60	12.50	15.96	14.74	14.40	85.60
8165	MSH3-gRNA1	2	9	8.75	20.97	17.39	15.70	84.30
8165	MSH3-gRNA1	2	35	3.60	5.00	4.44	4.35	95.65
8165	MSH3-gRNA1	8	38	13.85	19.39	16.25	16.49	83.51
8165	MSH3-gRNA1	9	32	9.55	6.36	12.87	9.59	90.41
8165	MSH3-gRNA1	1	17	6.15	2.94	1.90	3.67	96.33
8166	MSH3-gRNA1	5	87	17.21	11.11	15.87	14.73	85.27
8166	MSH3-gRNA1	5	126	11.61	14.17	19.70	15.16	84.84
8166	MSH3-gRNA1	3	84	5.05	11.39	5.61	7.35	92.65
8166	MSH3-gRNA1	1	26	6.85	8.33	7.41	7.53	92.47
8168	MSH3-gRNA1	8	53	25.00	29.33	30.30	28.21	71.79
8168	MSH3-gRNA1	5	68	12.24	5.00	12.77	10.00	90.00
8168	MSH3-gRNA1	6	38	10.00	7.84	6.86	8.24	91.76
8168	MSH3-gRNA1	2	18	12.31	18.92	16.82	16.02	83.98
8168	MSH3-gRNA1	8	38	14.17	19.18	11.54	14.96	85.04
8168	MSH3-gRNA1	6	83	8.27	9.57	10.39	9.41	90.59
8168	MSH3-gRNA1	2	33	10.00	9.80	14.47	11.43	88.57

8168	MSH3-gRNA1	6	44	9.09	11.76	14.29	11.71	88.29
8168	MSH3-gRNA1	8	1	18.30	18.35	16.94	17.86	82.14
8168	MSH3-gRNA1	7	15	16.28	8.57	13.25	12.70	87.30
8168	MSH3-gRNA1	5	4	15.38	21.74	25.53	20.89	79.11
8168	MSH3-gRNA1	4	3	16.00	29.27	35.48	26.92	73.08
8168	MSH3-gRNA1	4	0	86.25	84.85	86.67	85.92	14.08
8168	MSH3-gRNA1	8	2	23.66	28.43	32.84	28.31	71.69
8168	MSH3-gRNA1	3	0	22.00	28.57	36.96	29.18	70.82
8168	MSH3-gRNA1	5	0	100.00	100.00	100.00	100.00	0.00
8168	MSH3-gRNA1	12	86	20.87	13.85	24.53	19.75	80.25
8168	MSH3-gRNA1	3	16	15.96	18.29	16.36	16.87	83.13
8168	MSH3-gRNA1	4	14	20.97	14.81	28.00	21.26	78.74
8168	MSH3-gRNA1	7	74	11.54	5.13	8.70	8.45	91.55
8168	MSH3-gRNA1	4	7	21.21	21.57	26.09	22.96	77.04
8168	MSH3-gRNA1	2	24	22.50	14.56	23.53	20.20	79.80
8168	MSH3-gRNA1	11	87	18.87	13.33	10.45	14.22	85.78
8168	MSH3-gRNA1	5	3	21.50	28.17	25.32	25.00	75.00
8168	MSH3-gRNA1	3	9	11.93	9.78	17.78	13.16	86.84
8168	MSH3-gRNA1	4	14	23.44	24.68	32.26	26.79	73.21
8168	MSH3-gRNA1	8	67	7.84	6.48	7.04	7.12	92.88
8168	MSH3-gRNA1	11	118	23.53	9.45	13.13	15.37	84.63
8168	MSH3-gRNA1	6	18	30.00	28.05	29.58	29.21	70.79
8168	MSH3-gRNA1	2	23	3.57	5.19	6.86	5.21	94.79
8168	MSH3-gRNA1	5	23	6.62	6.40	5.43	6.15	93.85
8168	MSH3-gRNA1	12	55	11.11	14.29	14.89	13.43	86.57
8168	MSH3-gRNA1	6	77	5.37	3.96	7.08	5.47	94.53
8168	MSH3-gRNA1	4	27	3.85	5.74	7.94	5.84	94.16
8168	MSH3-gRNA1	5	28	5.32	5.56	9.41	6.76	93.24
8168	MSH3-gRNA1	8	72	11.54	19.78	15.45	15.59	84.41
8168	MSH3-gRNA1	8	32	9.40	8.46	13.04	10.30	89.70
8168	MSH3-gRNA1	2	1	12.07	12.68	12.32	12.35	87.65
8168	MSH3-gRNA1	4	19	5.45	4.44	4.55	4.81	95.19
8168	MSH3-gRNA1	11	35	11.82	11.59	14.29	12.57	87.43
8168	MSH3-gRNA1	7	70	13.66	10.48	16.38	13.51	86.49
8168	MSH3-gRNA1	2	12	10.00	13.27	10.53	11.26	88.74
8168	MSH3-gRNA1	3	5	13.24	16.46	11.54	13.74	86.26
8168	MSH3-gRNA1	3	0	4.00	2.68	2.86	3.18	96.82
8168	MSH3-gRNA1	1	20	1.63	2.86	1.49	1.99	98.01
8168	MSH3-gRNA1	5	48	4.00	5.41	3.74	4.38	95.62
8173	RSW4-gRNA1	4	144	4.17	1.82	1.29	2.43	97.57
8173	RSW4-gRNA1	1	23	2.74	1.69	2.82	2.42	97.58
8173	RSW4-gRNA1	3	72	12.28	7.69	13.51	11.16	88.84
8173	RSW4-gRNA1	2	48	13.56	10.53	19.67	14.59	85.41

8173	RSW4-gRNA1	5	134	4.32	6.92	7.50	6.25	93.75
8173	RSW4-gRNA1	2	63	1.06	0.75	2.25	1.35	98.65
8173	RSW4-gRNA1	3	86	1.15	1.90	1.09	1.38	98.62
8173	RSW4-gRNA1	4	125	1.50	1.37	1.32	1.40	98.60
8173	RSW4-gRNA1	4	103	11.24	18.52	31.91	20.56	79.44
8178	RSW4-gRNA1	7	172	1.05	3.64	2.00	2.23	97.77
8178	RSW4-gRNA1	3	87	1.80	1.94	1.96	1.90	98.10
8178	RSW4-gRNA1	3	107	1.43	1.65	1.90	1.66	98.34
8178	RSW4-gRNA1	3	54	1.89	2.78	2.17	2.28	97.72
8178	RSW4-gRNA1	5	181	1.04	2.56	1.02	1.54	98.46
8187	SYN4-gRNA1	3	81	4.17	3.17	2.82	3.39	96.61
8187	SYN4-gRNA1	6	131	2.97	2.73	2.48	2.73	97.27
8187	SYN4-gRNA1	3	65	1.12	3.45	2.44	2.34	97.66
8189	SYN4-gRNA1	7	133	1.64	1.77	1.42	1.61	98.39
8189	SYN4-gRNA1	7	151	0.74	1.67	1.77	1.39	98.61
8189	SYN4-gRNA1	2	42	1.20	2.94	1.80	1.98	98.02
8189	SYN4-gRNA1	5	101	1.59	1.85	2.22	1.89	98.11

Figure 11. Comparison of pollen viability between wild type Fielder plants (left panel) and MSH3 (middle panel) and RSW4 mutants (right panel). SYN4 was not shown as they were similar to wild type. Pollen grains that exhibit dense staining (using 2% acetocarmine) are indicative of viability, whereas pollen grains that are deformed, flaccid, and non-viable do not retain the stain.



8. Conclusions.

The identification of key genes controlling homologous recombination in canola and wheat was successfully achieved and a number of edited lines that manipulated these genes have been developed. These edited lines will be instrumental in understanding the specific role each of these genes plays in this essential process and hopefully should allow manipulation of the trait. There is still much learn from work in the crops in this important area, the polyploid nature of the crop genomes compounds the complexity of not only the trait but any research attempting to manipulate the trait. It became apparent during the course of the project that new and more efficient methods for assaying recombination levels need to be developed; this work is ongoing but more recently high throughput methods for both adaptive long read sequencing and direct pollen sequencing have been established and we believe that these methods offer promise for our work in the crop species.

9. List any technology transfer activities undertaken in relation to this project: *Include conference presentations, talks, papers published etc.*

All presentations and manuscripts that the project contributed to have (and will) acknowledged the source of funding.

Peer reviewed publications:

Higgins EE, Howell EC, Armstrong SJ, **Parkin IAP**. A major quantitative trait locus on chromosome A9, *BnaPh1*, controls homoeologous recombination in *Brassica napus*. New Phytol. 2021 Mar;229(6):3281-3293. doi: 10.1111/nph.16986.

Presentations:

I Parkin, E Dzananovic, P Gao, E Higgins, K Koh, A Sharpe. Detecting the genes controlling homologous recombination in *Brassica napus*. 16th International Rapeseed Congress, Sydney, Australia, September 26th, 2023. Selected Talk

I Parkin, C. Koh, E. Higgins, A. Sharpe. Uncovering the scope of fixed homoeologous recombination events in *Brassica napus* using long read sequence data. International Rapeseed Congress, Berlin, June 17th, 2019. Selected Talk

E Higgins*. Detecting de novo Homoeologous Recombination in *Brassica napus*. Plant and Animal Genome XXVII, Meiotic Recombination Workshop, January 14th, 2019, San Diego, US.
*Erin Higgins is a permanent biologist in Dr. Parkin's lab who assists with the project work.

V Bollina, Y Tan, CS Koh, P Bhowmik, E Higgins, T Orr, AMR Ferrie, **I Parkin**, AG Sharpe, **S Kagale**. Single Cell Genomic Sequencing in *Brassica napus*: Application in Monitoring Recombination Frequency. Plant and Animal Genome XXVII Conference. January 12-16, 2019.

Higgins, E, Clarke, W, **Parkin, I.** Homologous pairing control in Brassica napus. Plant and Animal Genome Conference, January 13-17th, 2018, San Diego, US.

Ongoing: Manuscript detailing meiosis in *Brassica napus* is has been submitted for peer review, we will update when we have a final decision on the manuscript. One additional manuscript is currently being drafted and will be submitted this year describing some of the edited lines. It is also expected that work describing the gene editing work in wheat will also contribute to a number of publications.

10. Identify any issues that arose during the project.

Impact of Covid-19 lockdown on the project progress: Due to provincial and federal regulations relating to the Covid-19 pandemic all personnel were prohibited from accessing the labs from mid-March 2020. We were given permission to resume active lab work in September of 2021; although we had reduced staffing levels to allow physical distancing, which necessitated a shift system in the lab. This also prohibited us from hiring summer student support.

Specific to the project: maintenance of our transgenic lines was deemed an essential task thus any material that was in the greenhouse or came out of tissue culture was cared for and seed/tissue was collected from these lines. However, we were unable to carry out any molecular characterisation or additional analyses of the developed lines during the lockdown, effectively for six months. The post-doctoral researcher working on the project was able to focus on analysing the *B. napus* gene expression

data during the enforced lockdown and a manuscript describing this work is being drafted. As indicated previously, the enforced delay will set back Objective 3; however, since our return to the lab, work has been progressing well and initial phenotyping of the lines has suggested meiosis has been impacted.

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