Canola Agronomic Research Program (CARP)
FINAL REPORT

The Final Report should fully describe the work completed for the year, noting the personnel involved and any deviations from the original plan and next and/or corrective steps as may be required if deviations are noted. A final financial statement summary of expenses must also be submitted. In the event of major changes within the budget, supporting notes are required. The final report is a summary of activity for the final year and an overview of the entire project.

Project Title: ***Drought tolerance in Canola through modulating the Kanghan (KH) gene family***

Research Team Information

Lead Researcher:		
Name	***Institution***	***Project Role***
Dr. Jitao Zou	National Research Council of Canada	Principal Investigator
Research Team Members (add rows as required)		
Name	***Institution***	***Project Role***
Wenyun Shen	National Research Council of Canada	Project activity lead
Hui Yang	National Research Council of Canada	Project team member-CRISPR gene editing

Project Start Date: April 1, 2022 **Project Completion Date:** April 1, 2024
Reporting Period: April 1, 2023 **to** April 1, 2024

CARP Project Number: **2022.13**

Instructions: This Final Project Report shall be completed and submitted on March 31th of the fiscal year that the agreement is in effect (upon completion of the project). The Lead Researcher of the project in question shall complete and submit the report on behalf of his/her complete research team.

This Report is a means by which to provide a detailed account upon completion of the project. Final project financial reporting should be provided at this time.

The following template is provided to assist you in completing this task. Please forward the completed document electronically to the CCC contact listed below.

In addition to the Final Research Report, a *Final Research Abstract/Extension Report* is due upon completion of the project, maximum 2-3 pages, to be used for publication on the Funders' websites and in the *Canola Digest*. Content will be used in extension material, for consumers and/or industry. Include an Executive Summary, brief project description, key findings and conclusions (with a summary graph/table or supporting image for the project), translation of key findings into best management practices and/or relevance to the canola sector and future research, and funding acknowledgment as determined in the grant award letter. The Final Extension Report is intended to support messaging to all audiences. Information needs to be clear, concise and in "grower-friendly"

language.

Please include the funding acknowledgements outlined in your research agreement in all deliverables (publications, presentations, etc.) from this project.

1. Date of completion & status of activity (please check one)

Date of completion: _____

☐ **Ahead of Schedule** ☐ **On Schedule** ☐ **Behind Schedule** ☑ **Completed**

Comments:

2. Summary - Maximum of one page. This must include project objectives, results, and conclusions.

We generated Canola RNAi lines in KH genes based on previous findings that KH mutations are associated with drought tolerance in Arabidopsis. We conducted field trial of the RNAi lines in Kelowna, BC, with "full irrigation" and "deficit irrigation" plots. In the 2 years periods, the trial season experienced above normal drought and heat conditions, and there were huge variations in yield within both "full irrigation" and "deficit irrigation" plots with results of two years experiment. At year 2022 growing season, indications of reduced yield loss under deficit irrigation were observed in some of the RNAi lines, but difference was not found at 5% level of statistical significance, depending on what statistics analysis methodology was implemented. At year 2023 growing season, the trial season experienced longer periods high heat condition, some indications of reduced yield loss under deficit irrigation were observed in the RNAi lines. The yield of some RNAi lines was higher than control line at almost all plots under deficit irrigation, but again, not found at 5% level of statistical significance.

We pursued CRISPR gene editing to generate KH homologs knockout lines in Canola. A large number of T0 plants harboring the gene knockout construct was generated, among which mutations in two different KH gene members have been confirmed through direct sequencing of the targeted genes. A total of 32 plants from the T1 generation of each mutation line were examined, leading to the verification of some homozygotes. Notably, gene mutations were confirmed in BnaA08g12920D, BnaC03g77550D, and BnaC03g77540D in T1 plants. However, mutations in BnaA08g12940D and BnaC03g77520D did not manifest in the T1 plants. Homozygous plants from the T1 generation were intercrossed and subsequently crossed with the commercial lines Stellar and Reston. The resultant hybrid seeds were harvested, representing essential materials for advancing gene function studies and facilitating breeding efforts focused on enhancing canola drought tolerance.

3. Introduction – Brief project background, rationale, and objectives.

Through a drought sensitivity screening of Arabidopsis natural accessions, we discovered that one ecotype originally from Northern Europe, designated #95, was capable of maintaining vitality after extended exposure to drought treatments. A gene family, *Kanghan* (KH), underpinning drought tolerance was discovered though QTL analysis, and homologs of the KH gene family (BnKH) were identified from *Brassica napus*. We generated BnKH RNAi lines. Growth chamber assessment demonstrated that RNAi suppression of the KH gene family in Canola leads to drastically improved drought tolerance.

Our discovery on the critical role of KH genes in drought tolerance provided a technological basis for improving canola yield stability under the persistent challenge of moisture shortage in the Canadian prairies. The primary objective of this project was to assess the KH technology under field conditions and its potential for drought

tolerance trait breeding in Canola. Since modulating KH gene may be better achieved by CRISPR for targeted gene knocking-out stability, the second objective of this project was to generate CRISPR lines disrupting KH genes in Canola germplasm for drought tolerance breeding.

4. Methods – Include approaches, experimental design, methodology, materials, sites, etc. Major changes from original plan should be cited and the reason(s) for the change should be specified.

The project includes two activities: activity one is to conduct field trial with the KH RNAi lines; and activity II is to generate CRISPR gene editing in KH homologs in Canola

A. Field trial

- This trial was conducted in Kelowna, BC surrounded by an apple orchard in an open field. The site was chosen due to the historically high heat and low natural precipitation experienced in the Okanagan Valley. The 2nd year's field trial was conducted at the same site. The field was rototilled prior to planting and sprayed with Bonanza for pre- emergent weed control.
- Six of RNAi lines and one control line (DH12075) was selected for the field trials (Table 1). We initially planned to use two different control lines. However, since DH12075 is the parent from which the RNAi lines were derived, we decided to use this line only as it would provide the most comparable genetic background to the RNAi lines. The plots were planted on July 6, 2022, and May 9, 2023, respectively.
- For the experiment design of both years, there were 6 sections for field trials (the trial maps shown in Fig.1, 2022 growing season, and Fig. 2, 2023 growing season); each section has 2 subsections, one was "full irrigation" subsection (subsection A, green color), and another "deficit irrigation" subsection (subsection B, blue color), close by at the same site of the slope. Each subsection has 7 treatments (Tr), representing different testing lines. The details of plant lines corresponding to different Tr are listed in Table1. The trial site is located on a gentle but conspicuously hilly slope. The soil is generally sandy in texture, with visible gravel and stone presence unevenly distributed in different sections of the slope. Disparity of soil quality is thus evident.
- A variable drip-line irrigation system was used to allow for controlled output of water to specific plots (i.e., partial irrigation and full irrigation). The initiation of the deficit irrigation treatments began at the 3-4 leaf stage on August 2, 2022 in Year 1 trail, at on June 9, 2023 in Year 2.
- Field assessments included stand counts, seedling vigor, chlorophyll measurements, NDVI (normalized difference vegetation index), leaf waxiness and cupping, leaf/plant wilting, days to start of flowering, days to end flowering, leaf senescence, count to determine branching, count of pods/plant, plant height, lodging, days to maturity, yield components (weight, moisture), thousand seed weight, as well as seeds oil and protein content.

Table 1. Treatment, RNAi lines and water application		
Treatment	RNAi lines	Water application
Tr 1	WT (12075)	Full irrigation
Tr 2	2191	Full irrigation
Tr 3	2192	Full irrigation
Tr 4	2193	Full irrigation
Tr 5	4971	Full irrigation
Tr 6	4972	Full irrigation
Tr 7	4975	Full irrigation
Tr 8	WT (12075)	Deficit irrigation
Tr 9	2191	Deficit irrigation
Tr 10	2192	Deficit irrigation
Tr 11	2193	Deficit irrigation
Tr 12	4971	Deficit irrigation
Tr 13	4972	Deficit irrigation
Tr 14	4975	Deficit irrigation

Figure 1. The field trial design and trial site picture of 2022 trial

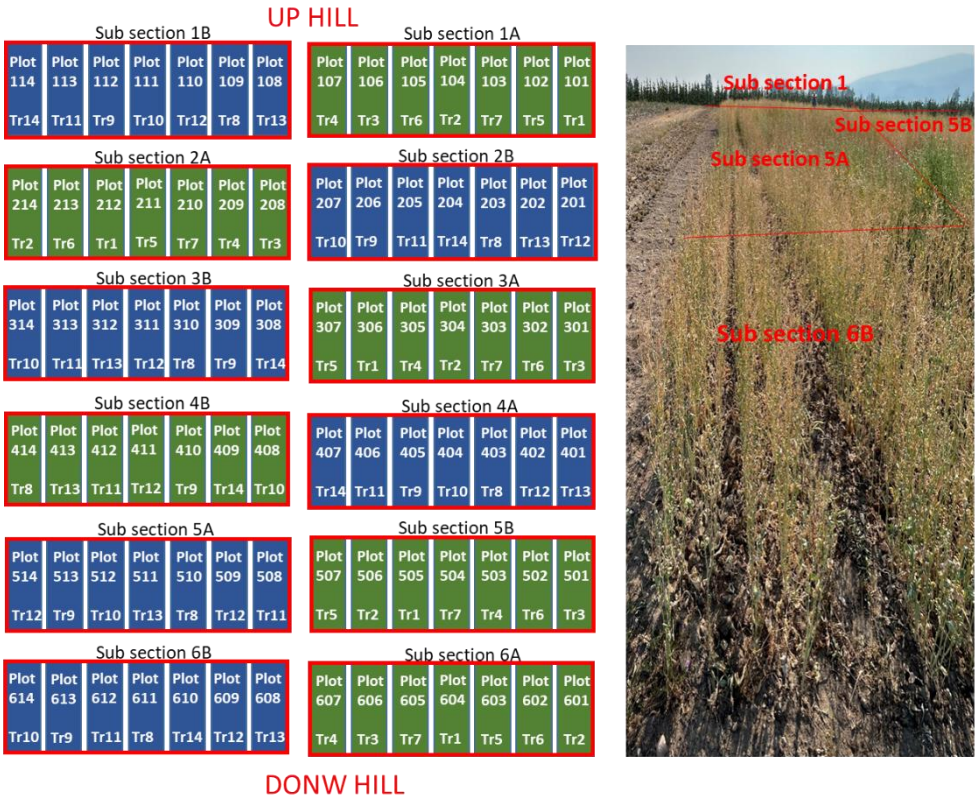

Figure 2. The field trial design and trial site picture of 2023 trial

B. Methods for generation of KH CRISPR gene editing lines

- A genome-wide exploration of the KH gene family across multiple *Brassicaceae* species, for which complete genome sequences are available, was conducted. KH homologs in A. thaliana, A. lyrata, A. helleri, B. napus, B. oleracea, and B. rapa were identified, and their protein sequences were used to construct a phylogenetic tree (see Figure 3). Pairwise analyses were performed to determine the closest homologs for each member across different species. Once their phylogenetic relationships were confirmed, CRISPR/Cas9 knock-out designs were developed to target various combinations of homologs in B. napus. Initially, nine genes were selected for targeting in the first stage: BnaA08g12920D, BnaC01g07670D, BnaC03g77540D, BnaA08g12930D, BnaC03g77550D, BnaC03g77520D, BnaA07g02270D, BnaA08g12940D, and BnaA01g06470D. Subsequently, BnaC01g08490D, BnaA01g07060D, and BnaC01g08520D were earmarked for later targeting.

- To fulfill the requirement of targeting multiple members of the KH gene family, we opted for a multiplexed toolkit (Cermak et al., 2017) and tailored it for application in B. napus. This toolkit is capable of accommodating up to 12 guiding RNAs (gRNAs), enabling the knockout of multiple target genes within a single construct. The strategic use of fewer constructs holds the potential to reduce the costs associated with plant transformations and downstream molecular confirmation of gene editing. Targeted gRNA design involved the integration of multiple bioinformatic tools, aiming to cover a broad

range of KH homologs with a minimal number of gRNAs while mitigating potential off-target effects. gRNAs, designed to target both conserved and specific regions of KH family genes, were confirmed through a genome-wide SNP/indels screening to identify duplicates and homologs across different subgenomes (AA and CC).

- The final selection comprised six gRNAs, which were tandemly connected with Csy-type ribonuclease 4 (Csy4) for simultaneous expression under a Pol II promoter (refer to Figure 4). The assembly of these elements was achieved through Gibson assembly (Gibson et al., 2009), facilitated by a specific primer list (Table 2) (refer to Figure 5). The ultimate plasmid for plant transformation was constructed following the Golden Gate protocol, linking Cas9, the gRNA cassette, and selection markers into the pTRANS_220d backbone (refer to Figure 6). This binary vector, designed for T-DNA insertion, incorporates the neomycin phosphotransferase II (npt II) selection marker (refer to Figure 7).

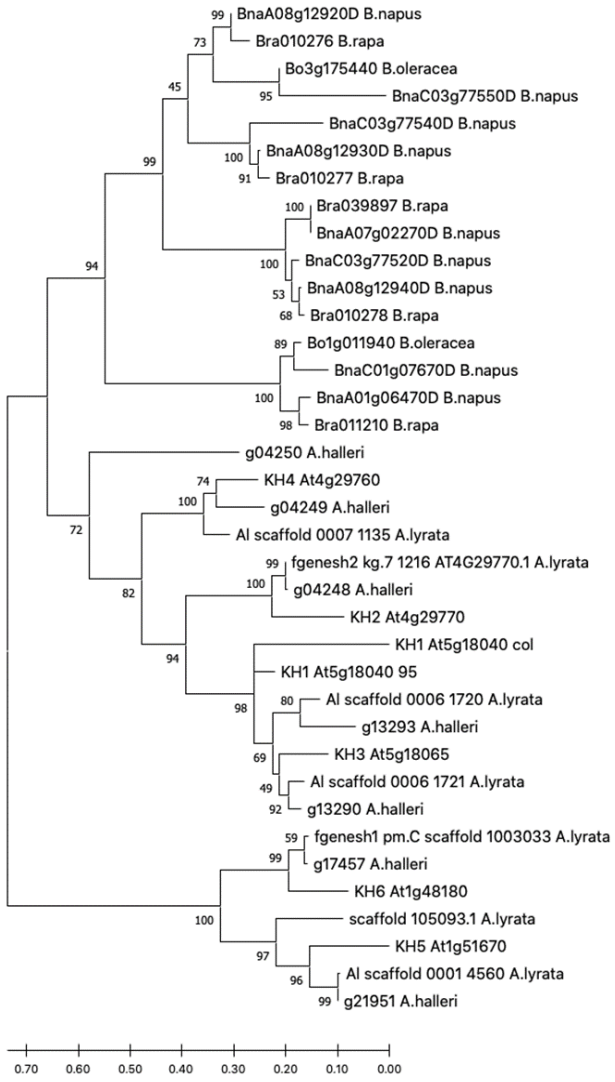

Figure3. Phylogenetic analysis of KH protein in diverse Brassicaceae species. Maximum likelihood (ML) phylogeny of KH homologs across different species based on Jones-Taylor-Thornton (JTT) model were constructed with 1000 bootstrapped replicates.

Figure 4. Cassette for simultaneous expression of four gRNAs.
A similar cassette will be used in this study containing 6 gRNAs in the final construct.

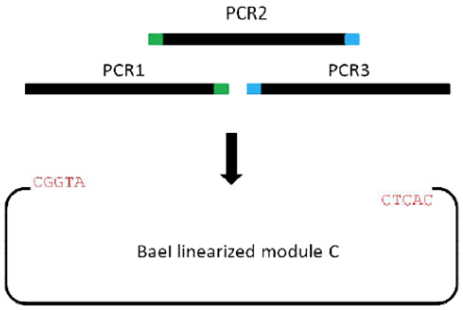

Figure 5. Overview of Cassette (See Figure 2) Construction Utilizing Gibson Assembly
This figure provides an overview of the cassette construction process (refer to Figure 2) employing Gibson assembly.

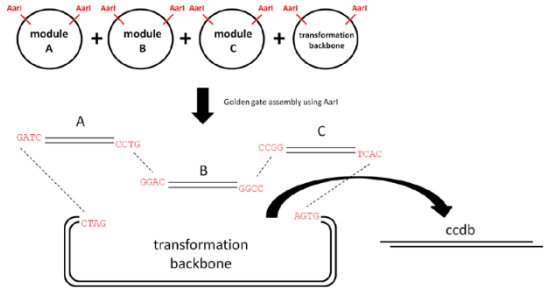

Figure 6. Construction of Plant Transformation Vector with Cas9 and gRNA Cassette
The plant transformation vector, encompassing the Cas9 and gRNA cassette, was constructed using the Golden Gate protocol.

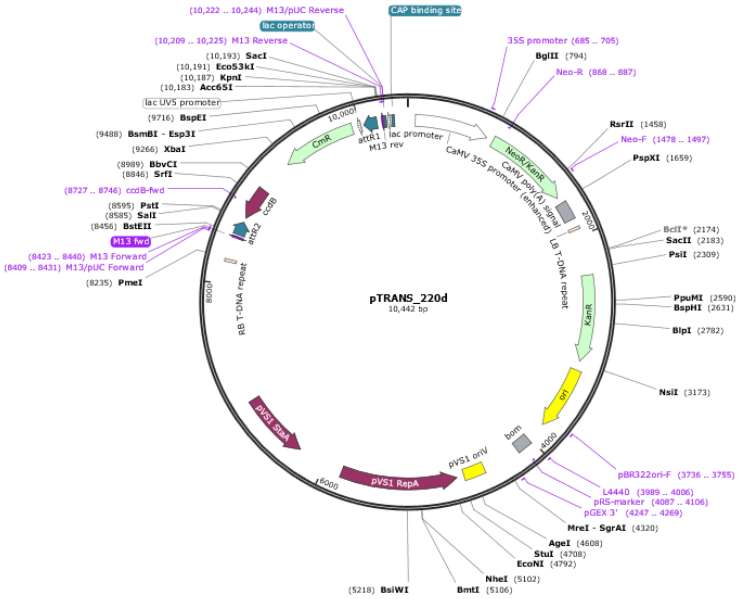

Figure 7. Construct Map for CRISPR/Cas9 Gene Editing of KH Family Genes
This figure illustrates the comprehensive map of the final construct designed for CRISPR/Cas9 gene editing targeting KH family genes.

Table 2. Primer list used for CRISPR plasmid construction of KH.

DG564	TGCTCTTCGCGCTGGCAGACATACTGTCCCAC
DG565	TCGTCTCCAGCGCACTCGAGCTGCCTATACGGCAGTGAAC
DG566	TCGTCTCACGCTTTCAAGGAGTTTTAGAGCTAGAAATAGC
DG567	TCGTCTCCCTTTGAAAGAAGCTGCCTATACGGCAGTGAAC
DG568	TCGTCTCAAAAGCGTACTCGGTTTTAGAGCTAGAAATAGC
DG569	TCGTCTCCCTCTCAGCAGAACTGCCTATACGGCAGTGAAC
DG570	TCGTCTCAAGAGAGCTGCTAGTTTTAGAGCTAGAAATAGC
DG571	TCGTCTCCGCCGAGTACTCGCTGCCTATACGGCAGTGAAC
DG572	TCGTCTCACGGCTCAGTTCCGTTTTAGAGCTAGAAATAGC
DG573	TCGTCTCCGCATTGGGCACACTGCCTATACGGCAGTGAAC
DG574	TCGTCTCAATGCTCTCTCCTGTTTTAGAGCTAGAAATAGC
DG575	TCGTCTCCACCATACGAGCACTGCCTATACGGCAGTGAAC
DG576	TCGTCTCATGGTAGCTAACCGTTTTAGAGCTAGAAATAGC
DG577	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC
HYCR01	TCGTCTCCTGGTATTGTGCGCTGCCTATACGGCAGTGAAC
HYCR02	TCGTCTCAACCAGACTCCGGGTTTTAGAGCTAGAAATAGC
HYCR03	TCGTCTCACAACTCGTTAATCTGCCTATACGGCAGTGAAC
HYCR04	TCGTCTCAGTTGGATGCCGGGTTTTAGAGCTAGAAATAGC
HYCR05	TCGTCTCACGGTATCCAACTCTGCCTATACGGCAGTGAAC
HYCR06	TCGTCTCAACCGGAGTCTGGGTTTTAGAGCTAGAAATAGC

5. Results – Present and discuss project results, including data, graphs, models, maps, design, and technology development.

Field trial Results

For the 2022 trial season, the plots were planted on July 6, 2022 and harvested on October 26, 2022. The seeding time was late in the season due to the time needed to complete field trial service contract and to secure CFIA filed trial permit at the specified sites prior to the seeding. For the 2023 growing season, the plots were planted on May 9, 2023 and harvested on September 1, 2023. The trial site is located on a hilly slope, and the soil is generally sandy in texture, with visible gravel and stone presence unevenly distributed in different sections of the slope. There is hence disparity in soil quality (Fig 8 left), which may caused a variable germination rate (Fig 8, right)

Figure 8. Soil condition and field statues of gemination

Three weeks after seeding while the plants had a few leaves (July 26-Aug 1), the trial site experienced one week of elevated heat, with ambient temperature at above 35 ^{o}C. In the 2nd year trial season, the summer daytime temperature experienced was also higher than what would be typically expected of Canola growing seasons in the prairies. The high temperature period was longer in the 2023 growing season as well. The precipitation in the both years were drier than normal.

Deficient irrigation treatment of some subsections started on Aug 2, 2022, and in Year 2, on June 9, 2023, at a time, when the plants were at a stage with 3-4 leaves. Each full irrigation subsection was accompanied by a deficit irrigation subsection, next to each other. The individual plots in the same subsection were relatively comparable in terms of soil conditions. Subsections at different areas of the slope had variable soil conditions.

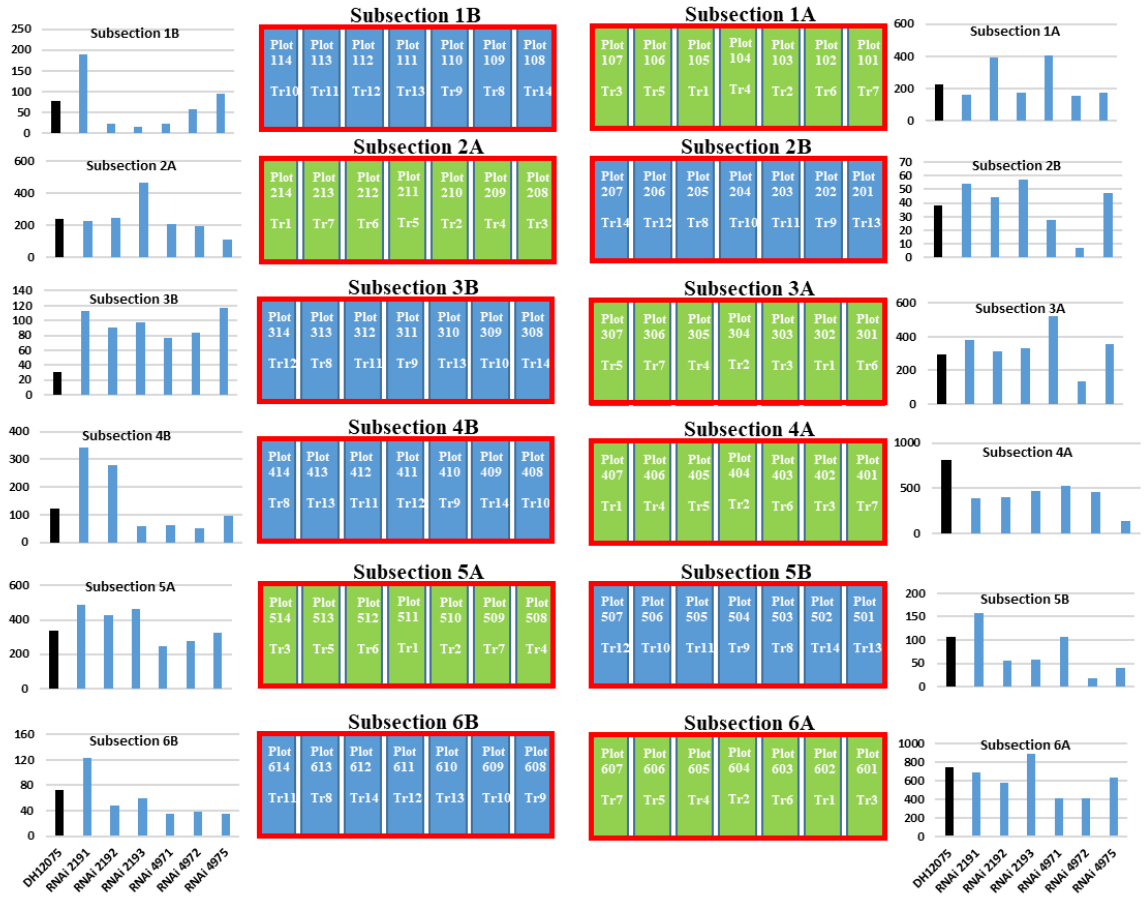

Figure 9. The results of yield per plot from each subsection (2022)

Charts in Fig 9. show yield data of 2022 growing season from each plot, adjusted based on germination rate. The first bar of each chart (black) presents yield of the control line (DH12075), although the location of the WT line in each subsection was randomized. The yield of the various RNAi lines in the same subsection are in blue.

Yield of different plots, even among the fully irrigated subsections, varied greatly. This partly reflected the challenge of drought field trial, and partly we think was attributable to soil condition variability of the field trial sites (Fig 8). When visually glancing through the yield of different lines raised close to each other in the same "deficit irrigation" subsections, there were indications that some of RNAi lines performed better than the control. Tr9 (RNAi2191), in particular, had a better yield in all of the "deficit irrigation" subsections. However, when subjected to Tukey's HSD analysis, which is a very conserved test, there was no statistically significant difference at $p<0.05$. Indication of better yield from Tr9 (RNAi2191) under deficient irrigation could only be found at $p<0.14$.

Given the huge variation of the yield, we performed outlier data identification using the statistics IQR (interquartile range) method. The plots with the most outliers were found to be from plot 4xx. We thus performed data transformation by removing plots in subsection 4A (Full irrigation, plot 401-407) and subsection 4B (deficit irrigation, plot 408-414). The datasets still have 5 replicates for each sample. HSD Tukey's test p-value of the "deficit irrigation" plots showed a difference at a $p < 0.1$. Test through other field trial statistic assessments, including the Fisher-LSD test with Benjamini-Hochberg (BH) multiple test correction, showed that Tr9 (RNAi2191) had a significant difference ($p=0.04679$) when

compared to the WT control (Tr8, DH12075) as shown in Figure 10, suggesting that this line had a significantly reduced yield loss under deficit irrigation conditions.

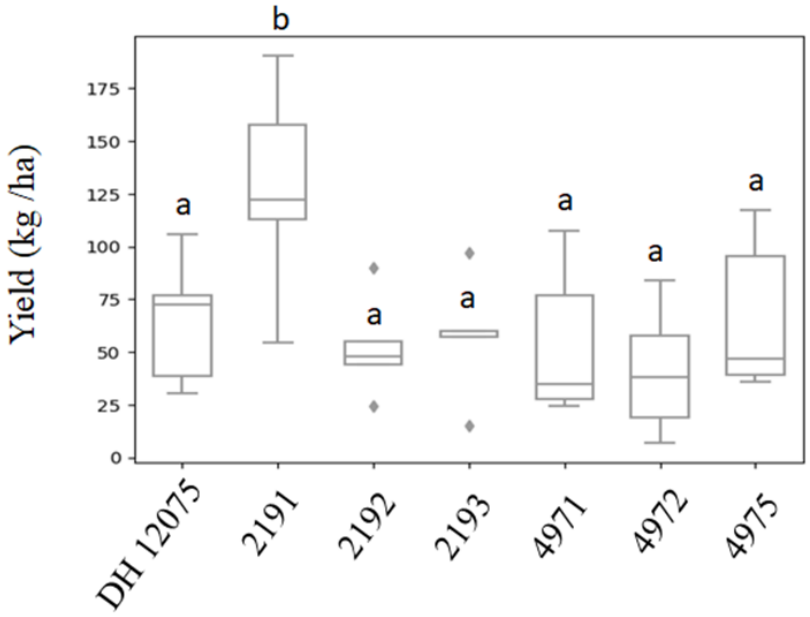

Figure 10. Statistic analysis of yield at deficit irrigated condition. The data presented as mean ± SE (n=5). Same small letter a to b indicate statistical difference with one way ANOVA followed by the Fisher-LSD test (p<0.05).

Other traits that may be related to yield were also examined at the 2022 growing seasons. There were significantly more pods per plot (5 plants) in the fully irrigated treatment (Trts1-7) than the deficit irrigation treatment (Trts8-14) except Trt 9. Trt8 (DH12075) had significantly fewer pods than Trt9 (Fig. 11 A), as well as significantly less branching than Trts 9 and Trt 12 (Fig. 11 B). These results were consistent with observations that Trt9 had higher yield than control line (Trt8) at deficit irrigation treatment (Fig.10).

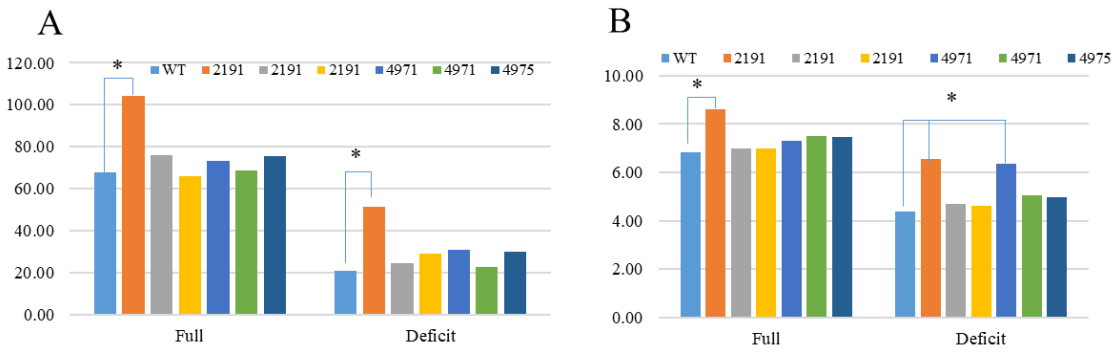

Figure 11. Number of pods (A) and branching (B) in each treatment (2022). The data presented as mean ± SE (n=6). Two-tailed Student's t test; *P < 0.05; **P < 0.01; ***P < 0.001

There were no significant changes at seed oil content in deficit irrigation compare with full irrigation, except the Trt 4 (RNAi2193). However, seed protein content was significantly higher in plots deficit irrigation than those of full irrigation (Fig.12).

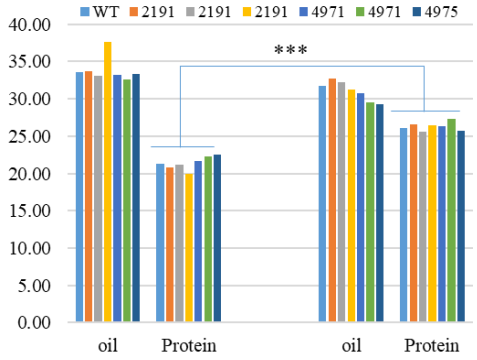

Figure 12. 2022 seeds Oil and protein Content. The data presented as mean ± SE (n=6). Two-tailed Student's *t* test; *P < 0.05; **P < 0.01; ***P < 0.001.

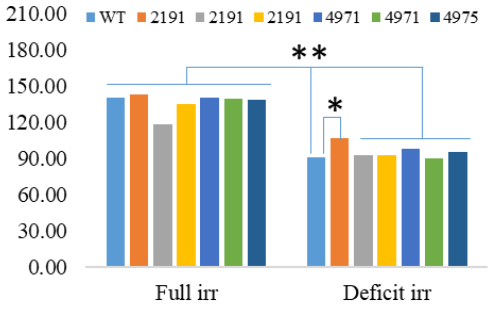

Figure 13. Plant high (2022). The data presented as mean ± SE (n=6). Two-tailed Student's *t* test; *P < 0.05; **P < 0.01.

At 5 weeks post initiation of deficit irrigation, lines at full irrigation plots exhibited significantly higher NDVI, leaf waxiness, and lower leaf wilting than the same lines under deficit irrigation regime. The plant height was significantly taller at full irrigation lpots except Trt9. Trt9 was significantly taller than control line (Trt8) at deficit irrigation (Fig. 13),

The days of start flowering were somewaht longer in some of the RNAi lines when compared with control line, regardless under full or deficit irrigation, but the difference was not statistically significant (Fig 14 A). The days of end of flowering was significantly longer at full irrigation than deficit irrigation (Fig 14 B).

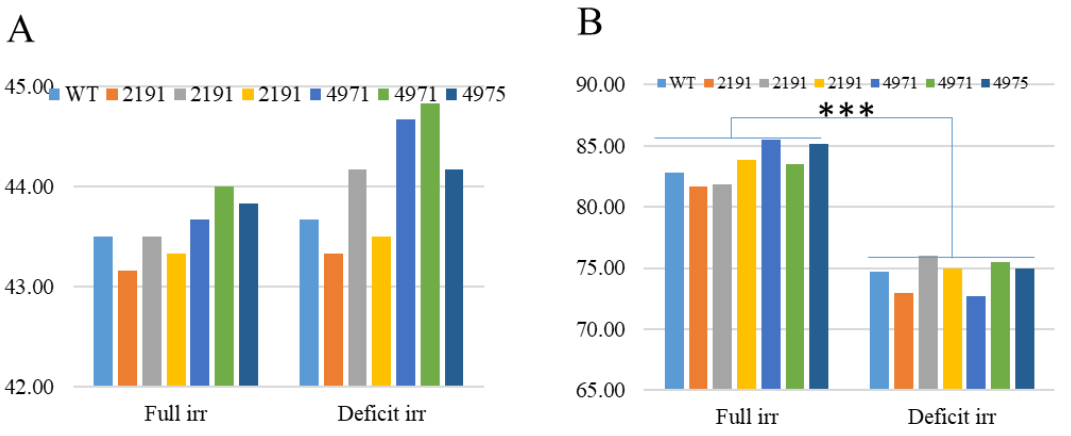

Figure 14. Days to start (A) and end (B) of flowering (2022). The data presented as mean ± SE (n=6). Two-tailed Student's *t* test; *P < 0.05; **P < 0.01.

The 2023 growing season was also dry, and the heat stress period was longer. The plots were planted on May 9, 2023 and harvested on September 1, 2023. Deficient irrigation treatment of some subsections started on June 9, 2023. At this time, the plants were at a stage of 3-4 leaves. Each full irrigation subsection was accompanied by a deficient irrigation subsection, next to each other. The individual plots in the same subsection were relatively comparable in terms of soil conditions. Subsections at different areas of the slope had variable soil conditions.

Charts in Fig.15 show yield data of the 2023 growing season from each plot. The first black bar presents yield of the control line (DH12075, Trt8), although the location of the control line in each subsection was randomized. The yield of the various RNAi lines in the same subsection are in blue.

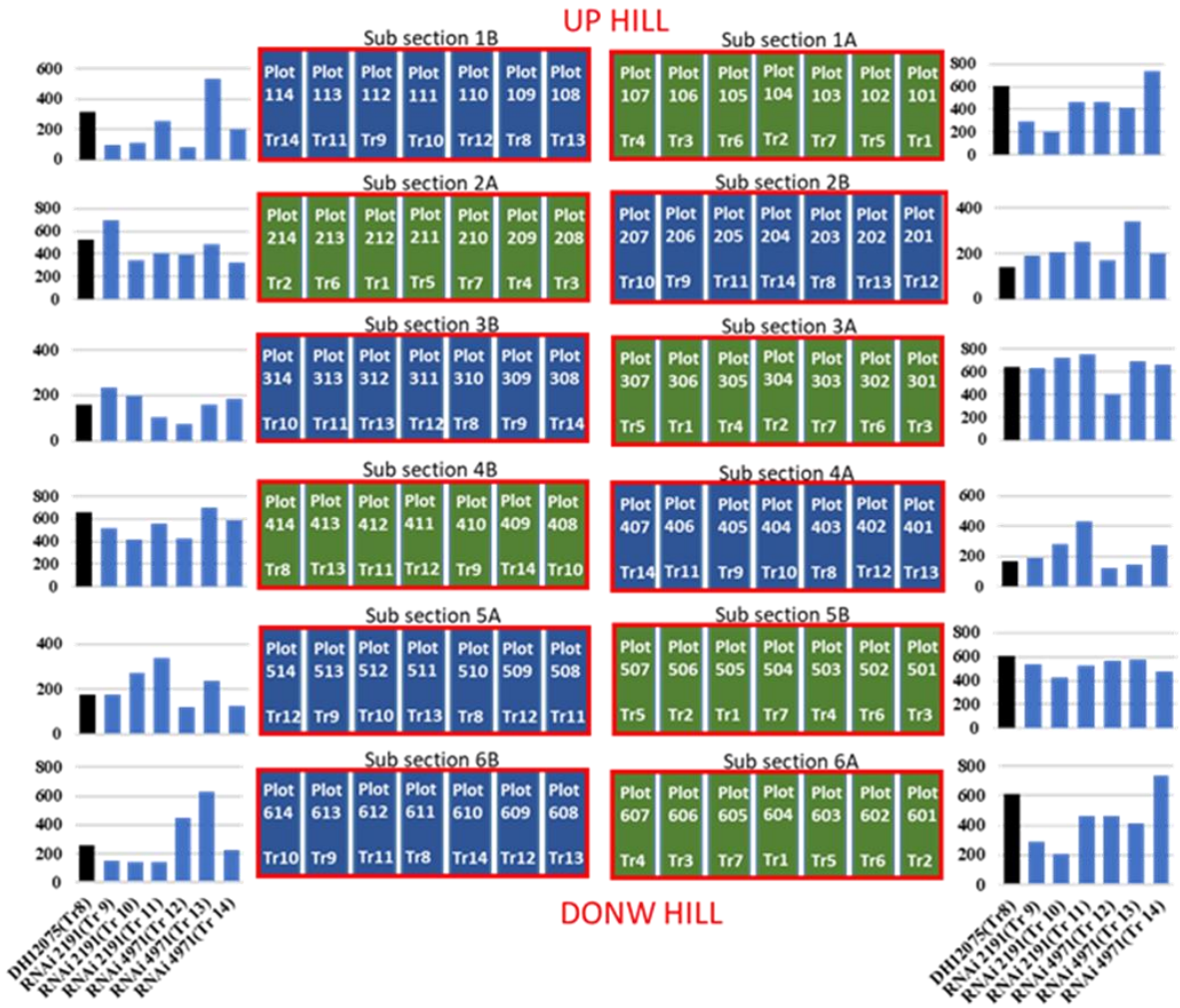

Figure 15. The results of yield per plot from each subsection (2023)

Similar to the Year 1 trial, when visually glancing through the yield of different lines raised close to each other in the same "deficit irrigation" subsections, there appears to be indications that some of RNAi lines performed better than the control. Tr13 (RNAi4972), in particular, had a better yield in 5 of the "deficit irrigation" subsections. However, when subjected to Tukey's HSD and Fisher-LSD test with Benjamini-Hochberg (BH) analysis, there was no statistically significant difference at $p<0.05$ (Fig 16).

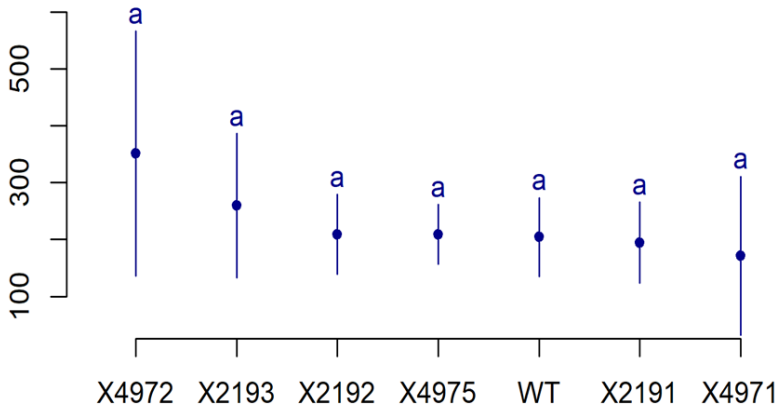

Figure 16. Statistic analysis of yield at deficit irrigated condition. The data presented as mean ± SE (n=6). Same small letter a indicate no statistical difference with one way ANOVA followed by the Fisher-LSD test (p<0.05).

There were no significant changes at seed oil content in deficit irrigation when compared with full irrigation. However, same with the Year 1 trail, seed protein content was significantly higher from the deficit irrigation plots (Fig.17).

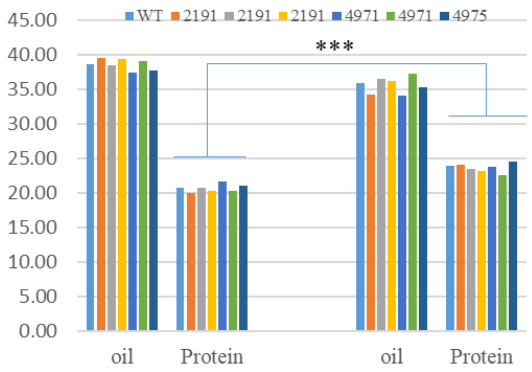

Figure 17. 2023 seeds Oil and protein Content. The data presented as mean ± SE (n=6). Two-tailed Student's *t* test; *P < 0.05; **P < 0.01; ***P < 0.001

No significant differences in stand count, vigor, chlorophyl content, waxiness, cupping, were detected between irrigation regimes and treatments. At 3 weeks after initiation of deficit irrigation, the NDVI was significantly higher, and the wilting was significantly lower in the full irrigation than deficit irrigation, as expected.

The days of start of flowing was longer in RNAi lines when compared with control line at both full and deficit irrigation. The days of start of flowering was significantly longer in lines 2193, 4971 and 4975 than

control line at full irrigation (Fig 18. A). The days of end of flowering was significantly shorter at deficit irrigation (Fig 18. B).

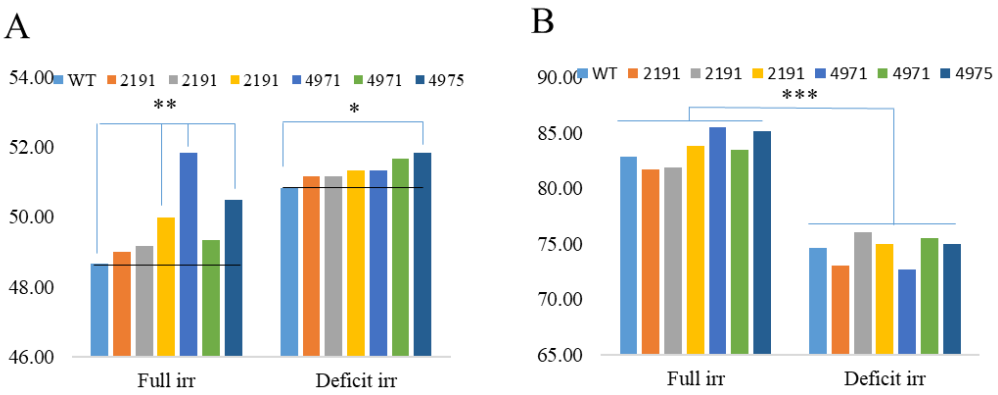

Figure 18. Days to start(A) and end (B) of flowering (2023). The data presented as mean ± SE (n=6). Two-tailed Student's *t* test; *P < 0.05; **P < 0.01.

Pods number, plant height, and biomass were significantly lower under deficit irrigation, but not statistically significant between lines at same irrigation regime at 2023 growing season.

Generation of KH CRISPR Gene editing lines

- **Generation of Transgenic Plants via Agrobacterium-Mediated Transformation.**
  The transformation process was executed in the canola cultivar DH12075, utilizing the CRISPR/Cas9 construct generated through agrobacterium-mediated hypocotyl transformation. Positive transformants were authenticated in T0 generation transgenic lines using a pair of npt II specific primers. A total of 57 independent lines with positive transformations were successfully generated. Additionally, a new Ti plasmid targeting three genes was introduced through Brassica napus hypocotyl transformation. The emergence of antibiotic-resistant green shoots indicates the successful initiation of the transgenic plant development process, and their full establishment is anticipated shortly.
- **Validation for the gene editing**.
  The Gene editing events were identified through direct sequencing or by cloning and sequencing PCR products spanning sgRNAs. Among the T0 lines, three exhibited mutations, each affecting a distinct target gene. The first line displayed a single-base deletion (G) in BnaA08g12920D (Fig 19b), while the second manifested a single-base deletion (G) in BnaC03g77550D (Fig 19a). The third line presented a triad of gene editing occurrences: a single-base insertion (A) in BnaA08g12940D (Fig 19e), a single-base insertion (A) in BnaC03g77520D (Fig19d), and a combined 2-base (CA) insertion and 3-base (AGA) deletion in BnaC03g77540D (Fig 19c). To augment the number of gene-edited plants, the T1 population of these mutation lines underwent screening. A total of 32 plants from the T1 generation of each mutation line were examined, leading to the verification of some homozygotes. Notably, gene mutations were confirmed in BnaA08g12920D, BnaC03g77550D, and BnaC03g77540D in T1 plants. However, mutations in BnaA08g12940D and BnaC03g77520D did not manifest in T1 plants.
- **Crossing with gene mutation lines and Canola commercial lines.**
  Homozygous plants from the T1 generation were intercrossed and subsequently crossed with the commercial lines Stellar and Reston. The resultant hybrid seeds were harvested, representing essential materials for advancing gene function studies and facilitating breeding efforts focused on enhancing canola drought tolerance.

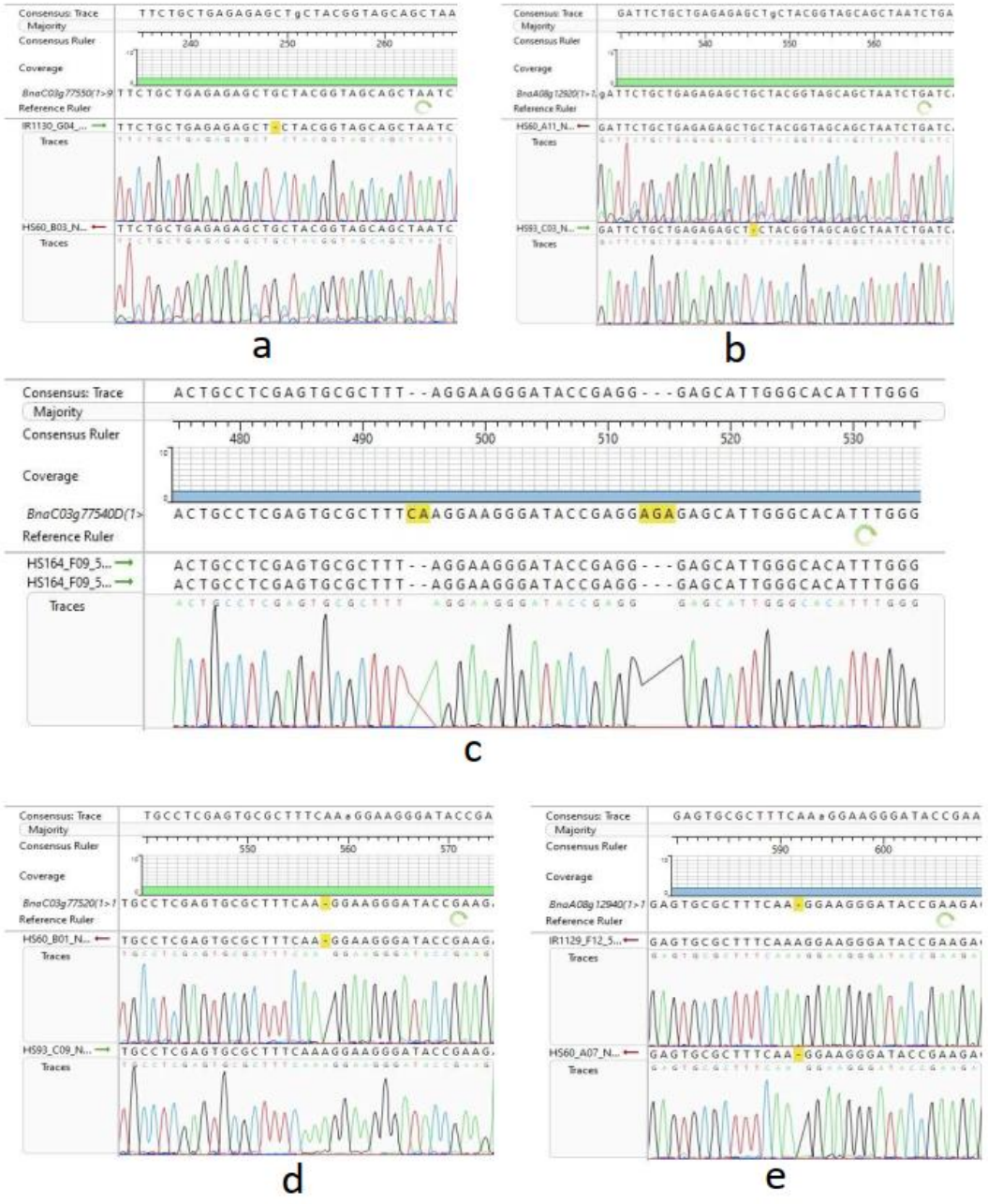

Figure 19. CRISPR KH Gene Editing with Various Mutations
(a) Single base deletion (G) in BnaC03g77550D; (b) Single base deletion (G) in BnaA08g12920D; (c) 2-base (CA) and 3-base (AGA) deletion in BnaC03g77540D; (d) Single base (A) insertions in BnaC03g77520D; (e) Single base insertion (A) in BnaA08g12940D. Yellow indicates the mutation positions within the gene.

- **Drought tolerance of T1 CRISPR lines**

  Drought tolerance in T1 CRISPR lines was conducted, primarily results show in the Fig 20, line 55-31

and 202-5-2 displayed a slightly better drought tolerance than WT control.

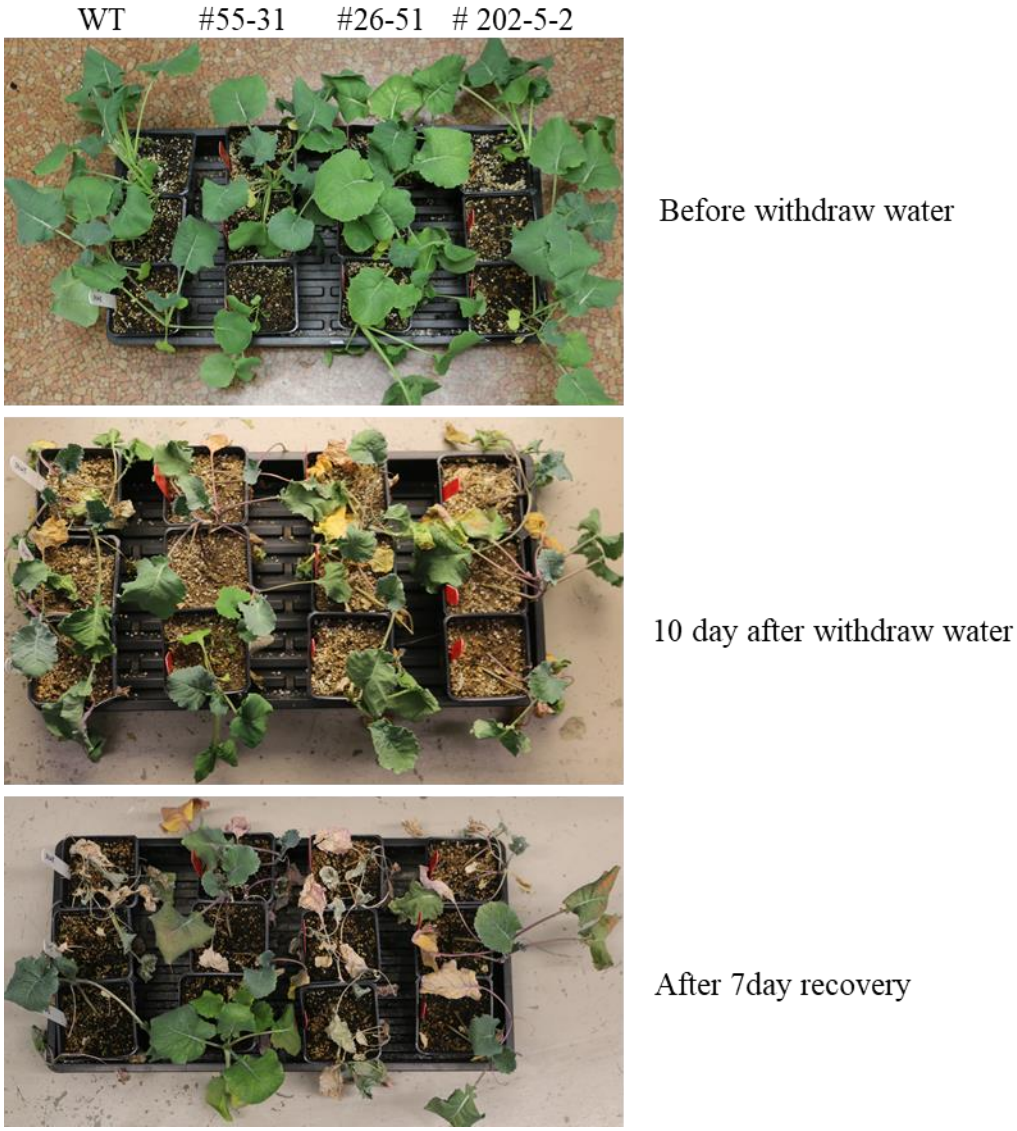

Figure 20. Evaluation of drought tolerance in T1 CRISPR lines.

6. Conclusions and Recommendations – Highlight significant conclusions based on the discussion and analysis provided in the previous section with emphasis on the project objectives specified above; also provide recommendations for the application and adoption of the project results and identify any further research, development, and communication needs, if applicable.

The field trial of the KH RNAi Canola lines was conducted in Kelowna, BC, where the last 2 years experienced not only drought but also above normal heat conditions. The unevenness of soil quality at different sections of the trial site added complexity to the experiments. But this issue was partly addressed by having 2 subsections close by in each section of the trial plot, one “full irrigation” subsection, and another “deficit irrigation” subsection. Adjustments of irrigation system were also made to mitigate factors causing variation in germination

rate. Given these technical challenges, we reasoned that comparing lines raised close to each other in the same "deficit irrigation" subsections would help interpretate the data. The yield of different lines raised close to each other in the same "deficit irrigation" subsections displayed a trend that some of RNAi lines performed better than the control. At year 2022 growing season, reduced yield loss under deficit irrigation were observed in some of the RNAi lines. However, when yield from all subsections were taken into consideration, the difference would not be found at 5% level of statistical significance if a most stringent statistics analysis methodology was implemented. From the 2023 growing season, indications of reduced yield loss were also observed in the RNAi lines, and in keeping with this, the yield of some of the RNAi lines was found to be higher than the control line at a majority of subsections. Nonetheless, the reduced yield loss was not detected at 5% level of statistical significance. It was also discovered during the trial that the RNAi line exhibited somewhat delayed flowering time, but the significance of that to yield is unknown. Conclusive yield performance trial generally requires much longer trial than 2 growing seasons. It is also noteworthy that both growing seasons witnessed heat stress much sever than what would be typically expected from prairie Canola growing regions, and during the 2023 growing season, the trial season experienced unusually longer period of high heat condition. It is unknown whether the added heat stress affected the yield performance of RNAi lines.

We have generated CRISPR gene edited KH knockout lines in Canola. Gene mutations were confirmed in BnaA08g12920D, BnaC03g77550D, and BnaC03g77540D in T1 plants. Homozygous plants from the T1 generation were intercrossed and subsequently crossed with the commercial lines Stellar and Reston. A number of mutants harboring mutations in two different KH gene members have been verified through direct sequencing of the targeted genes. Only preliminary drought tolerance assessment was conducted under controlled environment. The resultant hybrid seeds were harvested, representing essential materials for advancing gene function studies and facilitating breeding efforts focused on enhancing canola drought tolerance.

7. Extension and communication activities: (e.g. extension meetings, extension publications, peer-reviewed publications, conference presentations, photos, etc).

We are currently preparing a manuscript reporting our research in the KH gene discovery, tentatively entitled**: Enhanced drought tolerance conferred by naturally occuring loss-of-funtion mutations in a multi-member gene family,** which we expect to submit in June 2024.

8. Acknowledgements – Include actions taken to acknowledge support by the Funders.

We will fully acknowledge the support from CARP in our upcoming manuscript.

9. Literature Cited

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- Song, J.M. et al. (2020). Eight high-quality genomes reveal pan-genome architecture and ecotype differentiation of Brassica napus. Nat. Plants 6: 34–45.

10. Other Administrative Aspects: HQP personnel (PhD and/or MSc students) trained and involved; equipment bought; project materials developed
11. Appendices - If necessary, include any materials supporting the previous sections, e.g. detailed data tables, maps, graphs, specifications.
12. Financial (to be provided to each Funding Agency (at the addresses indicated in 11.2) a. Comprehensive Financial Statement that summarizes the total income and expenditures to date attributable to the Funders' Funding. b. Explanation of variances from budget which are greater than 10%. c. Invoice

13. Final Report Posting Do you consent to a version of this Final Report (with sensitive information removed) to be posted on the funder's website?	☑ Yes - this version can be posted ☐ Yes - a modified version will be sent ☐ No
14. Research Abstract Posting Do you consent to the 2-3 Research Abstract submitted with this Final Report to be posted on the funders and the Canola Council of Canada's website?	☑ Yes ☐ No

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