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Project title: Dissecting the association of flowering time and yield in oat
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Annual Status Report: Research

Summary

Flowering time is a key determinant of adaptation, yield, and cropping-cycle fit in oat. Our project uses CRISPR/Cas9 gene editing to dissect the regulation and functional contribution of *VRN3*, the central flowering-time integrator gene, with focus on *VRN3D* due to its strong genetic association with heading date in hexaploid oat. In the previous reporting period we completed the identification of *VRN3D* regulatory sequences, designed and validated allele-specific CRISPR/Cas9 constructs for *VRN3A*, *VRN3C*, and *VRN3D*, and generated the first-ever CRISPR mutants in oat. This report summarizes progress made during the current reporting period (1 June, 2025 to 31 May 2026), including (i) development of an independent *in vivo* assay to further substantiate *VRN3D* transcriptional regulation, (ii) a second, independent transformation targeting *VRN3D* to generate additional mutant events, and (iii) progress on genotypic and phenotypic screening of *vrn3* triple mutants, including expression analysis of downstream flowering genes and an unexpected photoperiod-dependent flowering. Based on this progress, the Agriculture Funding Consortium has approved a one-year extension, which will allow us to complete recovery and full molecular and phenotypic characterization of stable, transgene-free homozygous *vrn3d* knockout lines, and to finalize the DLR assay, both of which will be presented in the concluding report. We continue to acknowledge the support of the Agriculture Funding Consortium for enabling this work.

a) Progress

Objective 1: Identifying regulatory sequences in *VRN3* (7D) gene and refining gene editing in oat

This objective was completed and reported in our previous annual report, in which electrophoretic mobility shift assay (EMSA) data demonstrated that the SBP-SPL transcription factor binds directly to a GTAC-containing SBP-binding motif within the *AsVRN3D* promoter. However, we have been further working on an independent *in vivo* line of evidence for this interaction.

So far, we have developed a dual-luciferase reporter (DLR) for a transient expression assay in *Nicotiana benthamiana*. A 1.3 kb fragment of the *AsVRN3D* promoter spanning the SBP-binding sites was cloned upstream of the firefly luciferase (FLuc) reporter gene, and this reporter construct was co-infiltrated with either a 35S::AsSPL effector construct or an empty vector (EV) control, alongside a constitutive Renilla luciferase (REN) internal control (Figure 1A). Leaf discs were imaged for luminescence 2 days post-infiltration (Figure 1B).

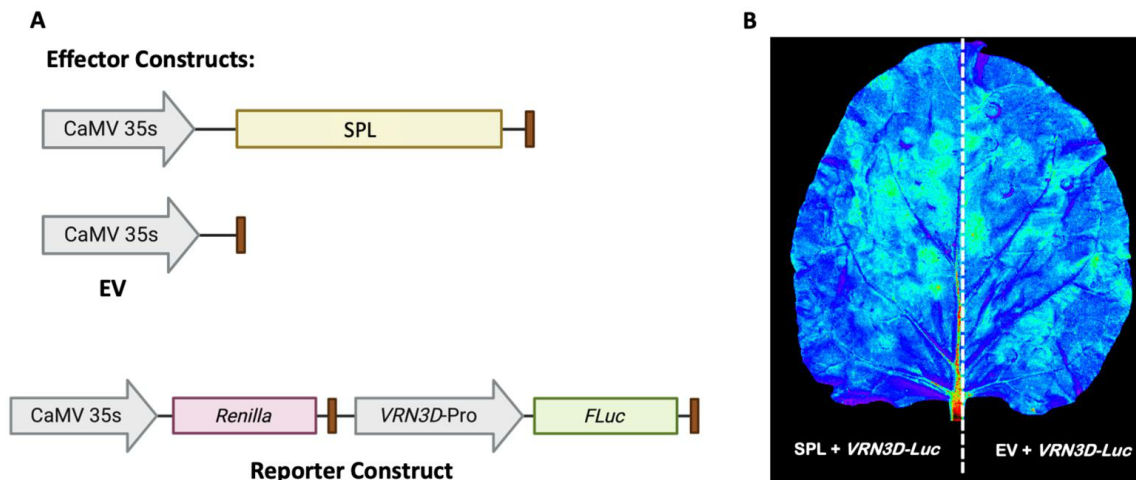


Figure 1. Standardization of the dual-luciferase reporter (DLR) assay for SPL–VRN3D promoter interaction. (A) Schematic of the effector constructs (35S::SPL and empty vector, EV) and the VRN3Dpro::FLuc / 35S::REN reporter construct used for *Agrobacterium*-mediated co-infiltration in *N. benthamiana*. (B) Representative luminescence image of a single infiltrated leaf, split between the SPL + VRN3D-Luc side and the EV + VRN3D-Luc control side.

This *in vivo* assay is intended to complement the EMSA data by testing whether AsSPL can activate the AsVRN3D promoter. We are currently expanding and refining signal quantification (firefly:Renilla ratio) to bring this assay to a statistically robust, publication-ready standard. Finalized, quantified DLR results will be presented in the concluding report next year.

Objective 2: Development of VRN3A/C/D allele-specific CRISPR/Cas9 constructs

This objective was completed in a previous reporting period, with allele-specific and pan-homoeolog CRISPR/Cas9 constructs (targeting VRN3D alone, and VRN3A/C/D jointly) designed, cloned, and validated for oat transformation, as previously reported. No additional construct design work was required or undertaken during the current reporting period.

Objective 3: Transformation of the said constructs into oat for developing mutant lines

Transformation of oat calli with the VRN3 and VRN3D-specific constructs, and regeneration of the resulting transgenic plants, was completed and reported previously. To generate an additional, independent cohort of VRN3D-edited events for homozygous line recovery, we carried out a second, independent round of biolistic transformation using the VRN3D-specific construct (pJDVRN3D) during the current reporting period.

Calli were selected on hygromycin-supplemented medium, and regenerated plants were screened for transgene integration by PCR amplification of the hygromycin (hpt) selectable marker gene.

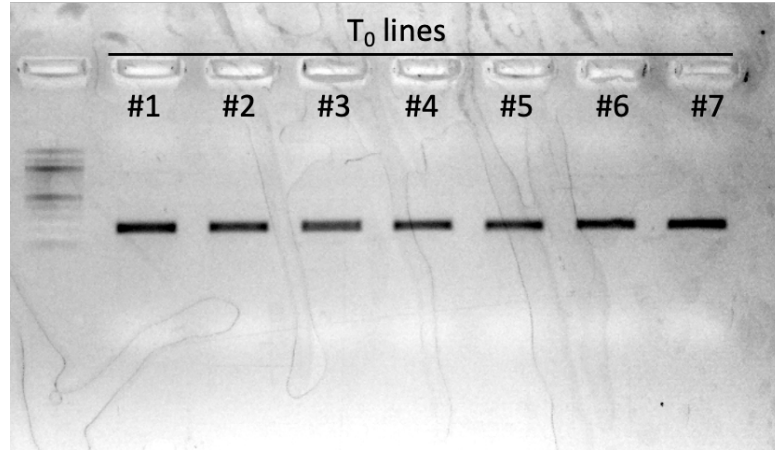


Figure 2. Hygromycin (hpt) PCR gel confirming transgenic events from the VRN3D-specific bombardment.

Regenerated, hygromycin-PCR-positive plants were transferred to soil and advanced for genotypic screening at the *AsVRN3D* target locus, described under Objective 4 below.

Objective 4: Genotypic and phenotypic screening of oat *VRN3A/C/D* mutants

A one-year extension has been granted to complete this objective. Currently, mutations in *VRN3A*, *VRN3C*, and *VRN3D* have been identified and validated at the molecular level, but recovery of stable, transgene-free homozygous *VRN3D* lines and their final phenotypic characterization remains outstanding. Substantial progress has been made on this objective during the current reporting period, summarized below.

4.1 Screening of the new VRN3D-specific mutant cohort (second bombardment)

Progeny from the second VRN3D-specific transformation (Objective 3) were genotyped by PCR amplification and Sanger sequencing of the region flanking the guide RNA target site. Sequencing traces show overlapping/dual peaks beginning at the expected cut site, consistent with a heterozygous editing event at the *AsVRN3D* locus rather than a fully resolved homozygous mutation (Figure 3).

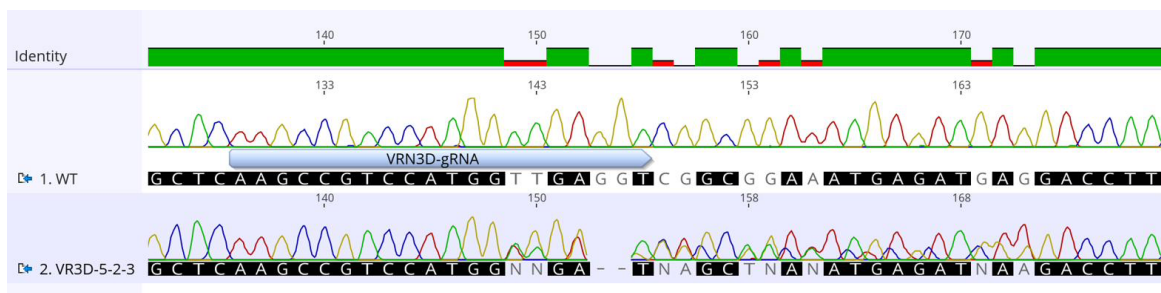


Figure 3. Sanger sequencing chromatogram at the *AsVRN3D* guide RNA target site for a representative line (VR3D-5-2-3) compared to wild type (WT). Overlapping peaks beginning at the cut site are consistent with a heterozygous genotype. Generation advancement and screening

of segregating progeny to identify stable, transgene-free homozygous lines is ongoing and will be completed for the final report.

This cohort is being advanced through subsequent generations, with segregants genotyped at each stage to recover homozygous, Cas9-free *VRN3D* mutant lines, which will then undergo the same phenotypic characterization pipeline described below for the triple-homoeolog mutant.

4.2 Expression analysis of *VRN3*-pathway genes in mutant lines

Using the published hexaploid oat transcriptome atlas (Avni et al., 2026), we examined tissue-specific expression of *VRN3*, *VRN1*, and *FT2* homoeologs, which showed the highest expression of all three gene families in internode tissue, with the D-genome copy consistently the most highly expressed homoeolog (Figure 4, heatmap). We then used quantitative real-time PCR (qRT-PCR) to compare relative expression of *VRN1* and *FT2* in internode tissue of wild type, a Cas9-free null segregant, and two independent *VRN3* mutant genotypes (*vrn3acd* triple and *vrn3cd* double mutants).

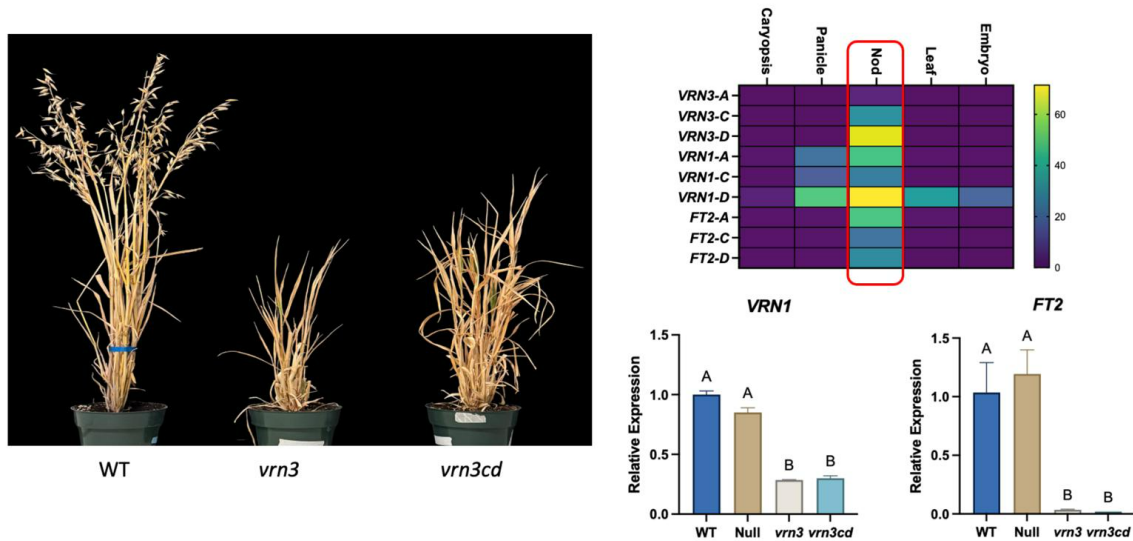


Figure 4. Expression of *VRN3*-pathway genes across wild-type and *VRN3* mutant genotypes. Left: representative mature plants of WT, *vrn3*, and *vrn3cd* showing a delayed/reduced reproductive phenotype in the mutants relative to WT. Top right: RNA-seq-derived heatmap of *VRN3*, *VRN1*, and *FT2* homoeolog expression across five oat tissues, with nodal tissue (boxed) showing the highest overall expression. Bottom right: qRT-PCR-based relative expression of *VRN1* and *FT2* in WT, a null segregant, and the *vrn3* and *vrn3cd* mutants. Letters denote statistically distinct groups (Tukey's post hoc test).

Both *VRN1* and *FT2* transcript levels were significantly reduced in the *vrn3* and *vrn3cd* mutants relative to WT and the null segregant (distinct statistical groupings, Figure 4), while WT and the null segregant were statistically indistinguishable from one another. This indicates that loss of *VRN3* function in these lines disrupts normal accumulation of downstream floral-integrator transcripts, consistent with *VRN3* acting upstream of *VRN1/FT2* in the oat flowering pathway.

4.3 A photoperiod-dependent flowering observation in the VRN3 triple mutant

As part of phenotypic screening, we also tested the *vrn3* triple mutant under different photoperiod regimes to investigate whether the delayed-flowering phenotype could be overcome by manipulating day length. Plants were grown under continuous long-day conditions (16 h light/8 h dark), continuous short-day conditions (8 h light/16 h dark), or an initial period of long-day growth (over one month) followed by a shift to short-day conditions.



Figure 5. Photoperiod-dependent flowering response of the *vrn3* triple-homoeolog mutant. Wild-type flowers normally under continuous long-day conditions (left). *vrn3* triple-mutant plants failed to flower under continuous long-day or continuous short-day conditions, but flowered when shifted from prolonged long-day growth (>1 month) to short-day conditions.

Mutant plants failed to flower under continuous exposure to either photoperiod alone. However, plants that were grown under long-day conditions for an extended period (over one month) and then shifted to short-day conditions did flower. This is an unexpected and interesting observation, suggesting that in the absence of functional *VRN3*, floral transition in oat may still be triggered by a shift in day length following a period of prolonged long-day exposure, rather than by either photoperiod in isolation. This raises the possibility of a *VRN3*-independent, photoperiod-shift-sensitive pathway contributing to floral induction in oat, which we plan to investigate further, including in the context of the newly recovered *VRN3D*-specific mutant lines (Section 4.1).

b) Remaining work and next steps

The main outstanding item for project completion is the recovery and final characterization of stable, transgene-free homozygous *VRN3D* lines from the second, independent transformation described in Section 4.1. We will continue generation advancement and genotypic screening of this population, and will pair confirmed homozygous lines with the same phenotyping pipeline (heading date, plant architecture, and photoperiod response) used for the triple mutant. We also

plan to finalize the dual-luciferase reporter assay (Objective 1) with quantitative reporting and improved signal accumulation. Both datasets, together with the *VRN3D*-specific homozygous line phenotyping, will be presented in the final report.

c) Materials and Methods

Dual-luciferase reporter (DLR) assay

The reporter construct (AsVRN3Dpro::LUC) was generated by cloning the 1.3 kb promoter region of AsVRN3D spanning the SBP-binding sites (GTAC) into the pGreenII-0800-Luc vector. The pSOUP helper vector was used to mobilize constructs into *Agrobacterium* strain GV3101. Leaves of 4-week-old *Nicotiana benthamiana* seedlings were infiltrated with the AsVRN3Dpro::LUC reporter construct combined with either an empty pRI101-AN vector (EV) or 35S::AsSPL as an effector. Infiltrated plants were kept in the dark for 12 hours before transfer to long-day conditions (16 h light: 8 h dark) at 24°C. Two days after infiltration, leaves were incubated with 1 mM D-luciferin potassium salt (GoldBio, St. Louis, MO, USA), and luminescence was detected using a CCD imaging system (ChemiDoc, Bio-Rad). Firefly (LUC) and Renilla (REN) luciferase activities are being quantified using the Dual-Luciferase Reporter Assay System (Promega) across additional biological replicates.

Biolistic transformation and plant regeneration

Biolistic transformation was performed using the BioRad PDS-1000/He system (Bio-Rad, Hercules, CA) at 1,100 psi. Immature embryo-derived callus (cv. Park) was pre-treated on osmotic medium (C' medium supplemented with 0.2 M mannitol and 0.2 M sorbitol) for 3 hours prior to bombardment. Gold particles (0.6 µm) were coated with plasmid DNA using a standard CaCl₂/spermidine precipitation protocol. Following bombardment, calli were recovered on C' medium under low light at 27°C for one week, then subjected to three successive rounds of selection on C' medium supplemented with 20 mg/L hygromycin B (Phytotech) under a 16h light/8h dark photoperiod at 27°C. Hygromycin-resistant calli were transferred to regeneration medium, and resulting shoots were rooted and hardened before transfer to greenhouse conditions.

Genotyping and mutant identification

Genomic DNA was extracted from transgenic plants at the 3–4 leaf stage using the urea extraction method (Chen and Dellaporta, 1994). Transgene integration was confirmed by PCR amplification of the hygromycin (hpt) gene using GoTaq Green Master Mix (Promega) with the following profile: 95°C for 2 min; 30 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 60 s; final extension at 72°C for 5 min. Editing outcomes at the AsVRN3D target locus were assessed by amplifying the guide-flanking region using Q5 High-Fidelity 2× Master Mix (New England Biolabs) followed by Sanger sequencing. Sanger chromatograms were interpreted using the Syntheso ICE v2 tool (Conant et al., 2022), and, where relevant, next-generation sequencing amplicon data were processed using CRISPResso2 (Clement et al., 2019).

RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA was isolated from internode tissue at the flag-leaf stage using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), with concentration and purity assessed by NanoDrop spectrophotometry. RNA was treated with DNase I (Promega) prior to cDNA synthesis using the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies). Quantitative real-time PCR

was performed on a Mx3000P system (Agilent Technologies). Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001) and normalized against the housekeeping genes EF1A and EIF4A (Yang et al., 2020), using two biological and three technical replicates per sample.

Phenotyping and growth conditions

Mutant and wild-type plants (cv. Park background) were grown in the research greenhouse under a 16h/8h light/dark photoperiod at 22°C/15°C day/night temperatures, with a complete N-P-K (20-20-20) fertilizer applied at germination and twice during vegetative growth. Heading date was recorded as days after sowing to panicle emergence on the main tiller. Relative plant height was calculated by dividing each plant's height by the mean wild-type height for the same growth run. Panicle length was measured at maturity, leaf number was recorded at flowering, and leaf width was measured post-flowering at the widest leaf region. A minimum of eight plants per genotype were used for phenotypic measurements. For the photoperiod-shift experiment, additional cohorts were grown under continuous long-day (16 h light/8 h dark) or continuous short-day (8 h light/16 h dark) conditions, or under long-day conditions for over one month followed by a shift to short-day conditions, with flowering monitored across all regimes.

Statistical analysis

Statistical analyses were performed using Student's t-test for comparisons between two groups and one-way ANOVA for comparisons among more than two groups, with $P < 0.05$ considered statistically significant. Following ANOVA, Tukey's post hoc test was used for multiple comparisons among group means, represented using a compact letter display.

d) Extension and communication activities: (e.g. extension meetings, papers produced, conference presentations made, photos)

Publications:

1. Avni, R., Kamal, N., Bitz, L., Jellen, E. N., Bekele, W. A., Angessa, T. T., Auvinen, P., Bitz, O., Boyle, B., Canales, F. J., Carlson, C. H., Chapman, B., Chawla, H. S., Chen, Y., Copetti, D., Correia de Lemos, S., Dang, V., Eichten, S. R., Klos, K. E., ... Mascher, M. (2026). A pangenome and pantranscriptome of hexaploid oat. *Nature*, 649(8095), 131–139. <https://doi.org/10.1038/S41586-025-09676-7>
2. Mehtab- Singh, Kaye, C., Kaur, R. and Singh, J. (2025) A highly efficient CRISPRCas9-based gene-editing system in oat (*Avena sativa* L.). *Plant Biotechnol. J.*, <https://doi.org/10.1111/pbi.70146>

Conferences and presentations

1. Mehtab-Singh, Tripathi, S., Kaur, R., Singh, J., (2026). CRISPR/Cas9-mediated Dissection of Flowering Time Regulation in Hexaploid Oat. Canadian Society of Soil Science and the Canadian Society of Agronomy Conference, June 20-25, 2026, University of Guelph, Canada **(Oral Presentation)**.
2. Tripathi, S., Mehtab-Singh, Kaur, R., Singh, J., (2026). A Novel SPL3-VRN1 Regulatory Module Governing Floral Transition in Oat. Canadian Society of Soil Science and the

Canadian Society of Agronomy Conference, June 20-25, 2026, University of Guelph, Canada **(Oral Presentation)**.

3. Mehtab-Singh, Tripathi, S., Ambalatharasan, A., Kaur, R., Singh, J., (2026). From Triticeae to Avenae: Translational Dissection of the miR156/SPL and *VRN* Modules to fine-tune flowering in Hexaploid Oat. International Wheat Congress, Bologna, Italy. **(Poster Presentation)**.
4. Cali, K., Kaur, R., Mehtab-Singh, Singh, J., (2026). Functional Characterization of Thaumatin-like Protein 8 Using the CRISPR-Cas9 System. Canadian Society of Soil Science and the Canadian Society of Agronomy Conference, June 20-25, 2026, University of Guelph, Canada **(Oral Presentation)**.
5. Mehtab-Singh., Tripathi, S., Kaur, R., Singh, J., (2026). Decoding the Genetic Network of Heading Date and Yield In Oat Using CRISPR/Cas9. Plant and Animal Genome (PAG 33) Meeting, San Diego, USA. **(Invited Talk)**.
6. Mehtab-Singh., Tripathi, S., Kaur, R., Singh, J., (2026). CRISPR/Cas9-Mediated Dissection of Flowering Time Regulation in Hexaploid Oat. Plant and Animal Genome (PAG 33) Meeting, San Diego, USA. **(Invited Talk)**.
7. Mehtab-Singh., Kaye, C., Kaur, R., Singh, J., (2025). Oat joins the CRISPR club: A Breakthrough for Oat Breeders, 15th July, Oat Day 2025, Ottawa Research and Development Centre, Agriculture and Agri-food Canada (AAFC) **(Invited Talk)**.

PI: Invited Talks:

1. *Small Grain Cereals using Gene Editing and Complementary Strategies*, Delhi University, South Campus, Delhi, India (March 6, 2026).
2. *Precision Breeding through Genomics and Gene Editing for Sustainable Agriculture*, Workshop on “Scaling Sustainable Agriculture through Digital Innovation”, Punjab Agricultural University, Ludhiana, Punjab (February 24, 2026).
3. *Smart Breeding and Genomic Innovations for Climate-Resilient Small Grain Cereals*, Research Horizons Seminar Series, McGill University, Canada (October 23, 2025).
4. *Gene Editing and Complementary Approaches for Climate-Resilient Small Grain Cereals*, Brandon Research and Development Centre, Agriculture et Agri-Food Canada (October 2, 2025) **(Virtual presentation)**.
5. *Potential of Smart Genetics for Sustainable Agriculture*, Atlantic Canada’s Inaugural Sustainable Agriculture, University of Prince Edward Island, Charlottetown, PEI, Canada, August 6, 2025 **(Keynote presentation)**.

Media Reports:

1. Interview with Ms. Claire Loewen, Scientists edit oat DNA for the first time, paving the way for healthier, more climate-resilient crops (June 2025)
<https://www.mcgill.ca/newsroom/channels/news/scientists-edit-oat-dna-first-time-paving-way-healthier-more-climate-resilient-crops-365655>
2. Scientists edit oat DNA for the first time, paving the way for healthier, more climate-resilient crops (2025)
<https://www.mcgill.ca/newsroom/channels/news/scientists-edit-oat-dna-first-time-paving-way-healthier-more-climate-resilient-crops-365655>
3. Genome editing: researchers at McGill University have successfully modified oat DNA (2025) <https://montreal.citynews.ca/2025/07/15/genome-editing-mcgill-university->

[oat-dna/](#)

4. ***Researchers Successfully Edit Oat Genes for the First Time (2025)**, BIOTECH UPDATES, ISAAA Inc. May 28, 2025, <https://www.isaaa.org/kc/cropbiotechupdate/ged/article/default.asp?ID=21365>
5. ***Scientists successfully edit oat DNA using for first time, enabling climate-resilient crops (2025)** Phys News, June 10, 2025, <https://phys.org/news/2025-06-scientists-successfully-oat-dna-enabling.html>
6. ***Researchers make astonishing discovery that could reshape \$900 million food sector: 'This breakthrough is incredibly important'** Yahoo News, July 24, 2025, <https://www.yahoo.com/news/articles/researchers-astonishing-discovery-could-reshape-113031143.html>
7. **Gene-edited oats in the cards? (2025)** The Western Producer, June 10, 2025, <https://www.producer.com/news/gene-edited-oats-in-the-cards/>
8. ***Genetically edited oats: Canada opens a new page in grain breeding (2025)**, AgroPortal, Ukraine, June 16, 2025, <https://ukragroconsult.com/en/news/genetically-edited-oats-canada-opens-a-new-page-in-grain-breeding/>

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e) References

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Yang, Z., Wang, K., Aziz, U., Zhao, C., Zhang, M. (2020). Evaluation of duplicated reference genes for quantitative real-time PCR analysis in genome unknown hexaploid oat (*Avena sativa* L.). *Plant Methods* 16, 138.