Annual Status Report: Research

Summary

Flowering time is crucial for plant adaptation, impacting yield and other traits within cropping cycles and growing seasons. Various genetic factors regulate the expression of genes to facilitate vegetative to reproductive transition in plants. One of such flowering time genes is *VRN3*. However, its transcriptional regulation and association with flowering and yield-related traits remained unravelled. CRISPR holds a great potential in understanding the gene function by precisely tweaking its genetic makeup and altering the expression. We are using the gene editing system to understand the association of flowering time and yield with *VRN3D*. The transgene free *VRN3D* mutant lines with variability in flowering time can also be used by the oat breeders to acclimate it to the changing agro-climatic conditions. We acknowledge the support of the Agriculture Funding Consortium for their funding support to dissect these important traits in oat.

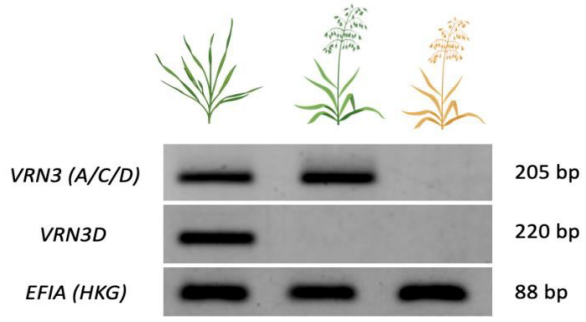
a) Progress:

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

There are different layers of gene regulation, one of which is at the transcription level. Transcription factors (TFs) are a huge group of regulators that control gene expression and act as on-off switches in regulation of various developmental processes (Liu et al., 1999). SQUAMOSA promoter binding like (SPL) proteins are plant-specific TFs responsible for the regulation of various processes associated with time of inflorescence development (Wang and Wang, 2015). In Arabidopsis, SPL3 regulates the *APETALA (AP1), LEAFY (LFY),* and *FRUITFULL (FUL)* genes that control the flowering time and floral induction (Yamaguchi et al., 2009).

Since *VRN3D* is also a flowering gene, we hypothesized and reported previously that a specific SPL transcription factor regulates its expression. Hence, the 2000bp upstream (promoter) region of the *VRN3D* gene was investigated for *cis-regulatory* elements. Interestingly, we found elements for various transcription factors including the SBP-SPL binding motif i.e., GTAC (highlighted in yellow). Biotin-labelled probes were designed for DNA-protein interaction analysis covering these binding sites.

Figure 1 . Semi-quantitative PCR of *VRN3 (A/C/D)* and *VRN3D* at different oat growth stages.



Transcriptional Regulation of *VRN3D*

The appropriate timing of flowering is one of the imperative components of optimizing the reproductive success of the plant. *VRN3D* is an important flowering gene that governs the vegetative to reproductive transition in cereals. However, the molecular pathway for its transcriptional regulation is poorly understood. Biotin-labelled probes were designed from the promoter region of oat *AsVRN3D* to observe its binding with the AsSPL3 protein. Our EMSA results indicate that probe Pi1 of *AsVRN3D* formed protein-DNA complexes with recombinant AsSPL3 and retarded their mobility on the gel. However, no such mobility shifts were observed with other probes (Pi4) designed from the *AsVRN3D* promoter (Figure 2). This binding reflects the role of AsSPL3 in the transcriptional regulation of *AsVRN3D*.

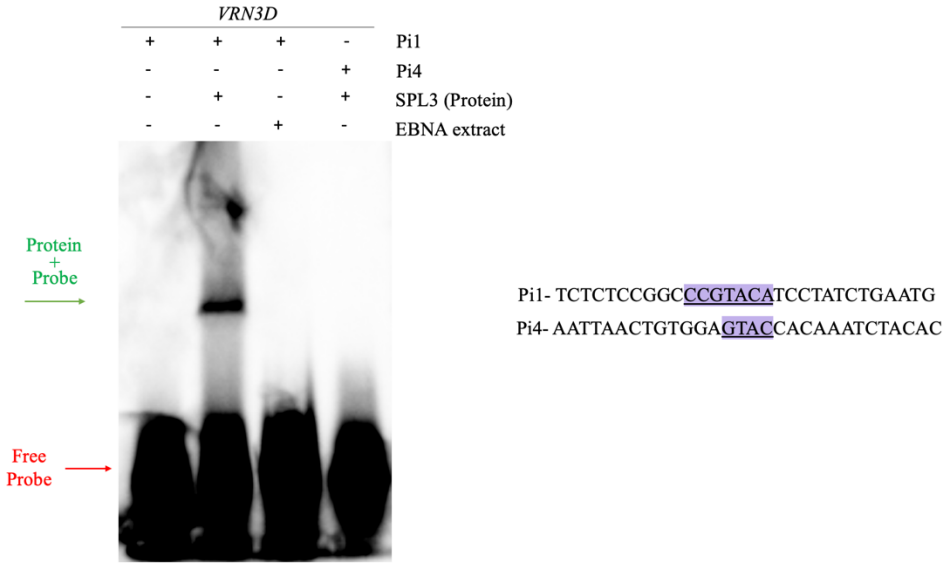


Figure 2. The interaction of AsSPL3 with oat *VRN3D*. (A) EMSA assay of *VRN3D* probes (Pi1 and Pi4) with the SBP domain of AsSPL3, and another protein (EBNA extract) as a negative control. - denotes the absence of a probe or protein indicated on the top of each lane. The SPL3 binding sites are highlighted in lilac on the right.

Material and Methods:

Electrophoretic Mobility Shift Assay (EMSA)

The EMSA assay was performed to analyze the interaction between the SPL3 transcription factor (protein) and the promoters of *VRN3* (DNA) using a non-radioactive EMSA kit (Thermo Fisher). The selected promoter regions of *VRN3D* were synthesized as biotin-labeled oligos and annealed to make double-stranded DNA. The binding reaction was prepared as per the manufacturer's protocol, and incubation was performed for 20 minutes at room temperature. Next, 5 µl of loading buffer was added to the 20 µl binding reaction, followed by thorough mixing using pipetting. To prepare a 6% polyacrylamide gel, 0.5X TBE buffer was used, and pre-electrophoresis was carried out at 100V with 0.5X TBE buffer before loading 20 µl of the samples onto the gel for electrophoresis. A universal transfer tank was used to transfer the gel onto the nylon membrane for approximately 30 minutes at 100V, and the transferred DNA was cross-linked using a UV light cross-linker for 60 seconds. Gentle shaking was done for 15 minutes after the addition of blocking

buffer to block the membrane, and the nylon membrane was then placed in a conjugate solution. Furthermore, the membrane was washed in 1X wash solution for 5 minutes and then transferred to the substrate equilibrium buffer. The nylon membrane was placed in a clean vessel wrapped with plastic, and the working solution was poured until the membrane was covered. It was incubated for 5 minutes without shaking. The moist membrane was wrapped with saran wrap and exposed to a CCD camera for photographing.

Objective 2 and Objective 3: Development of *VRN3A/C/D* allele-specific CRISPR/Cas9 constructs and transformation of the said constructs into oat for developing mutant lines

CRISPR Construct Design and Genetic Transformation using Biolistics

Different locations in the VRN3 gene were targeted for gRNA design. Separate guides targeting the *VRN3D* copy and all three *VRN3 (A/C/D)* copies were designed from the first exon (Figure 5). They were evaluated for the highest on-target and the least off-target scores to ensure the best efficiency and specificity of the CRISPR experiment. Following gRNA design various types of CRISPR constructs were designed to test the transformation efficiency in oat. pMV3D construct consists of single gRNA driven by barley U3 promoter targeting oat *VRN3D* gene. A total of 90 calli were bombarded with pMV3D that gave 5 transgenic events with transformation efficiency of 5.5% (Table 1). All the calli regenerated into plants but produced no edits (data not shown).

Two individual constructs (pJDVRN3, and pJDVRN3D) were designed by cloning the gene-specific guides driven by wheat U6 promoter in the JD633 backbone with ubiquitin promoters for GRF-GIF chimera, *Cas9*, and *hygromycin (hpt)* (Figure 3) (Debernardi et al., 2020). A total of 75 calli were bombarded with pJDVRN3 targeting the oat *VRN3*, yielding six transgenic plants confirmed through *hygromycin (hpt)* gene PCR with a mean transformation efficiency of 8% (Figures 4 and 5; Table 1). All the plants were successfully regenerated and transferred to soil in the growth chamber. Similarly, for the *VRN3D*-specific construct: pJDVRN3D, 77 calli were transformed, producing three transgenic plants with an efficiency of around 4%.

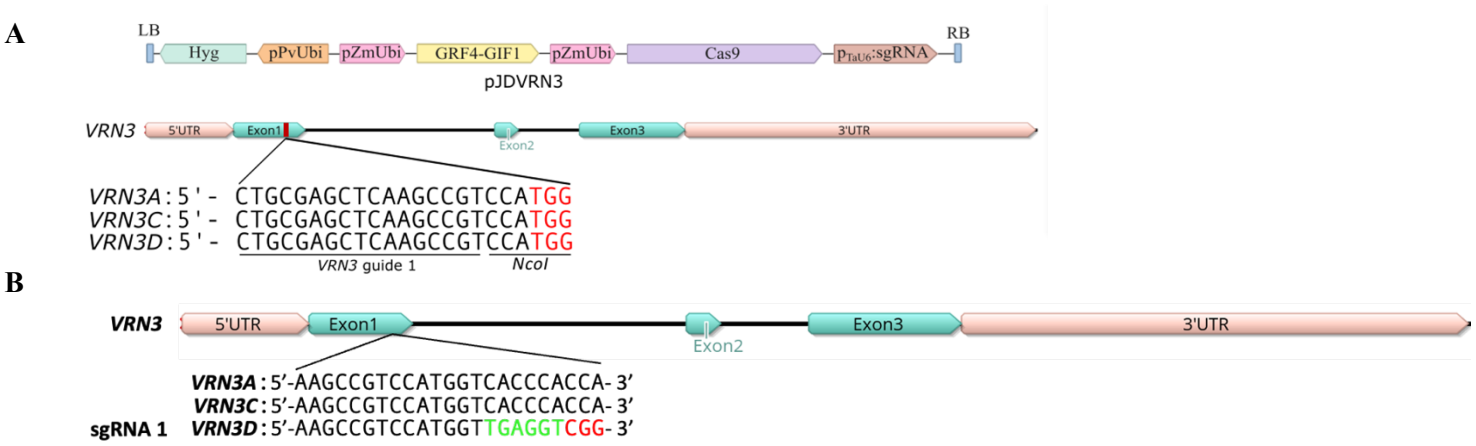


Figure 3. Targeted CRISPR/Cas9-mediated gene-editing in oat. A) CRISPR construct pJDVRN3 with *VRN3* gRNA from the conserved region. PAM is depicted in red. B) Depiction of VRN3D-specific gRNA used in pJDMV3D construct.

Table 1. Summary of *VRN3* transformations in oat.

S.No.	Construct	No. of cali bombarded	Transgenic cali	Transformation Efficiency (TE) (%)	Regeneration Efficiency (%)
2	pJDMV3D	77	3	3.8	100
3	pJDMV3	75	6	8.1	100

NA- Not applicable; TBD- To be declared.

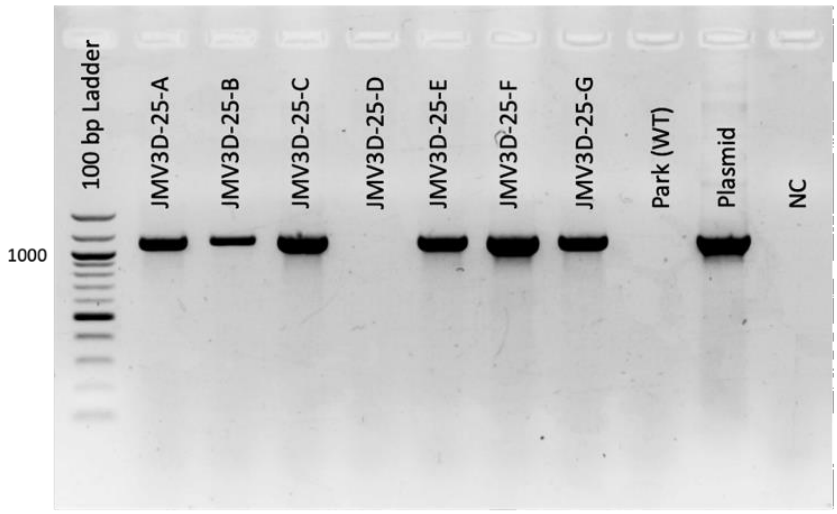


Figure 4. Successful transformants in oat confirmed using *Hyg* PCR.

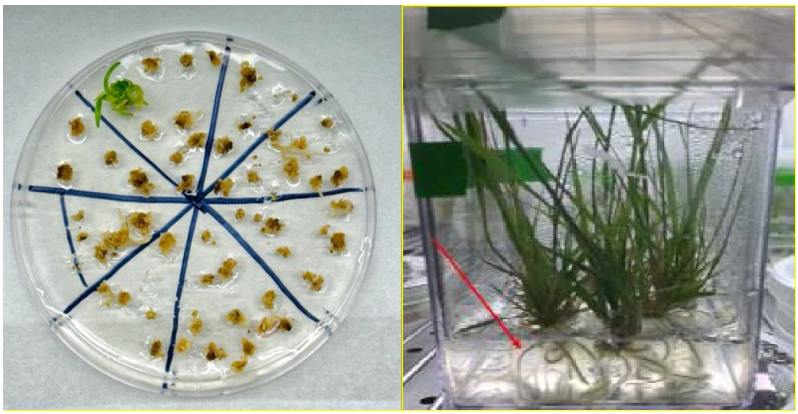


Figure 5. Transgenic calli selected on *Hygromycin* (Left); Transgenic plants rooting in the rooting media (Right)

Genotypic characterization of *VRN3* T_0 lines

The target region was PCR amplified from the T_0 transgenic lines using the *VRN3* primers (Appendix Table 1) and sequenced by Next Generation Sequencing. The sequencing results reported the first ever successful gene-edited oat plants with small deletions and insertions in the *VRN3* gene (Figure 6A). Intriguingly, the 4bp deletion in *VRN3D* altered the *NcoI* restriction site that facilitated the screening of knockout mutants through cleaved amplified polymorphic

sequence (CAPS) assay. The CAPS genotyping identified the gene-edited lines with 4bp deletion, depicting an undigested mutated PCR amplicon, while the WT control was completely digested (Figure 6C). The undigested amplicon was gel extracted, and a 4bp deletion was confirmed by Sanger sequencing (Figure 6D). For *VRN3A,* 3bp and 4bp deletions were reported as compared to an 8bp deletion and an A insertion in *VRN3C* (Figure 6A). In total, three gene-edited plants were obtained in the T_0 generation, with mutations in all three *VRN3* copies reporting a high gene editing efficiency of 50 % . We also generated VRN3D-specific gene-edited lines using the pJDVRN3D construct with a 20 bp deletion and a 2 bp deletion along with a T to G substitution (Figure 6B).

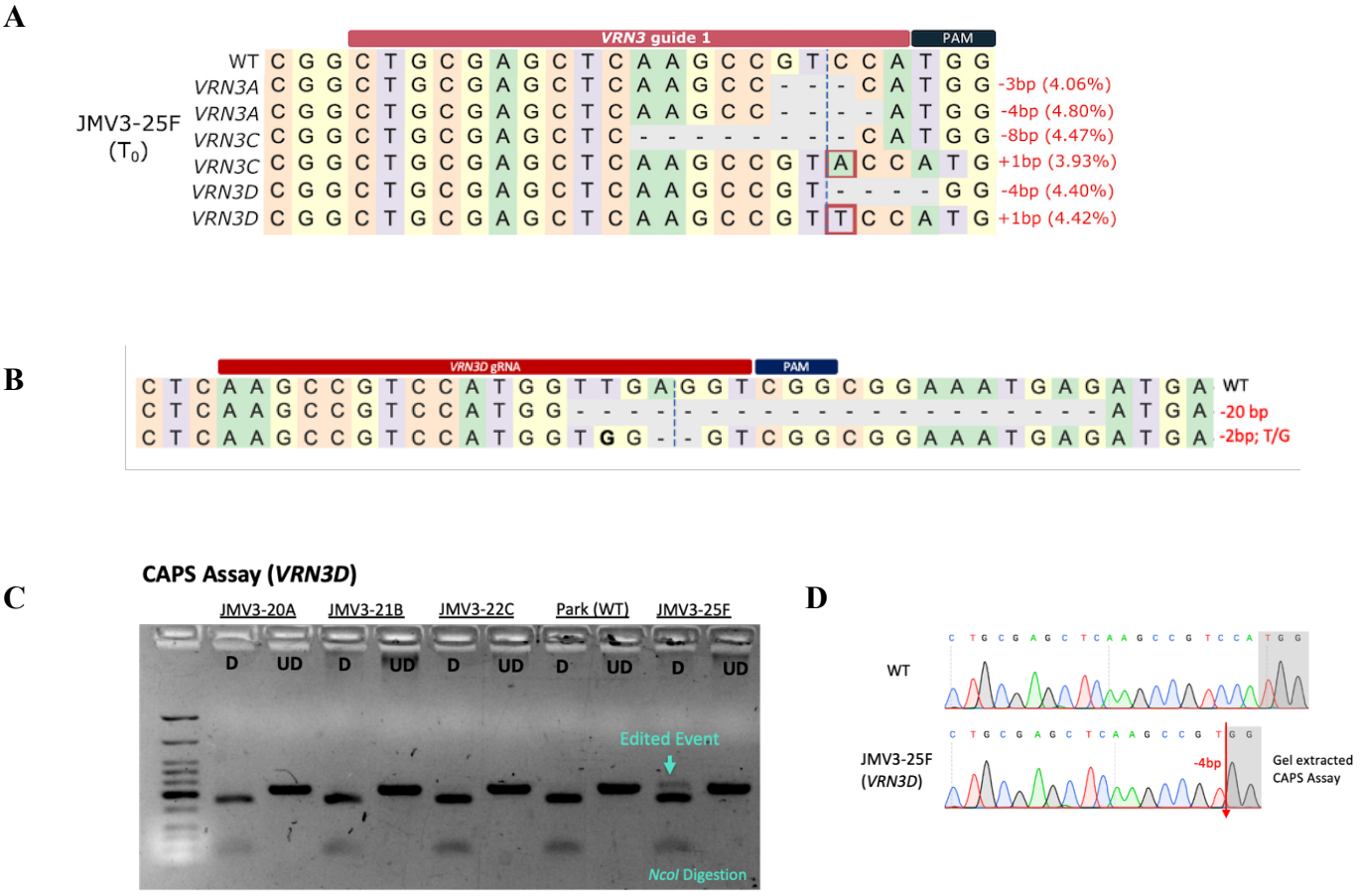


Figure 6. Targeted mutagenesis in the *VRN3* guide region. A) Deletions are shown with a dashed line and insertions with a red box leading to a frameshift mutation. Mutation types are indicated in red on the right. B) Targeted mutagenesis in the *VRN3D* guide region. Deletions are shown with a dashed line. Mutation types are indicated in red on the right. C) Cleaved amplified polymorphic sequence (CAPS) assay with *NcoI* for screening of gene-edited pJDVRN3 T_0 plants. D) WT and mutant chromatograms are shown at the guide region. PAM is highlighted in grey, and the deletions are depicted with a red arrow.

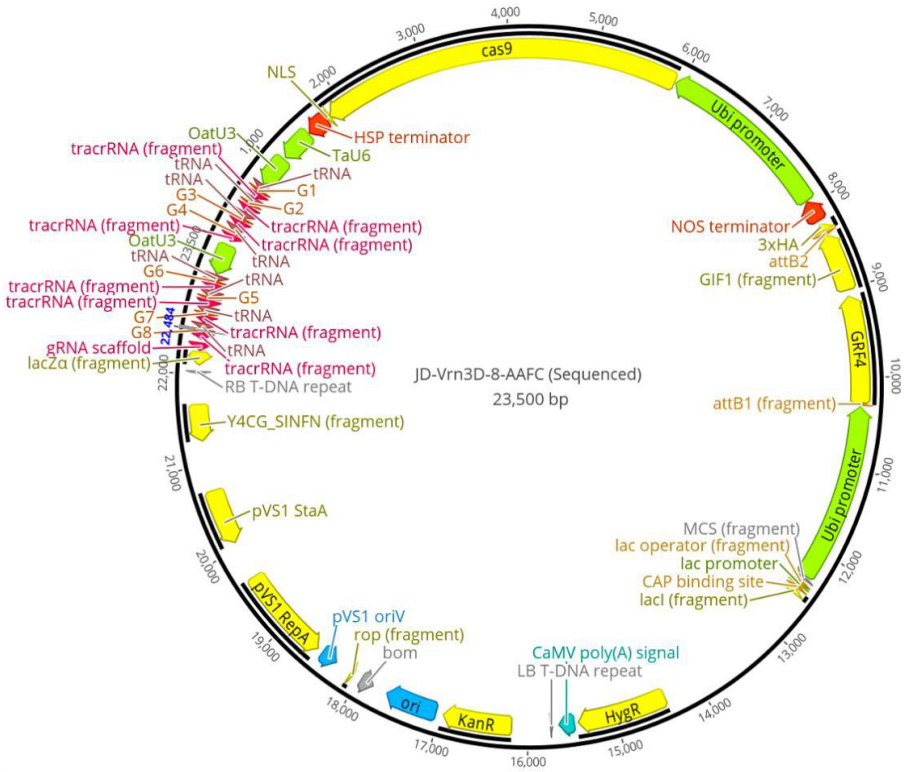
T_0 Generation						
Construct	**Gene**	**No. of Calli**	**Transgenic Plants**	**Transformation Efficiency (%)**	**Gene-edited Plants (Screened)**	**Gene-editing Efficiency (%)**
pJDVRN3	*VRN3*	75	6	8.0	3 (6)	50.0

T_1 Generation						
Construct	**T_0 Plant ID**	**Number of T_1 Plants**	**Gene-edited Plants**	**Cas9-free Plants:** 38 (58.4%)		
				Homozygous	**Heterozygous**	**Null**
pJDVRN3	25F	65	51 (78.5%)	2 (5.3%)	26 (68.4%)	10 (26.3%)

Table 2. Summary of transformation frequency, gene-editing efficiency and transgenerational inheritance.

Another way to target the *VRN3D*'s expression is by tweaking its promoter. In collaboration with AAFC, an improved version of JD633 was designed with an oat U3 promoter driving 8 gRNAs targeting various regions in the *VRN3D* promoter (Figure 7). Crop-specific promoters for driving gRNA have reported increased gene editing efficiencies (Ren et al., 2021). We recently transformed this construct and the calli are on the selection media.

Figure 7. pJDMV3D-Pro construct map with improved oat U3 promoter driving 8 gRNAs.



Genotyping and mutant identification

The genomic DNA was extracted from the T_0 and T_1 plants at (3-4 leaf stage) using the urea method (Chen and Dellaporta, 1994). The GoTaq® Green Master (Promega Corporation, Canada) was used to amplify the incorporated construct by *hygromycin* gene primers (1 µM). The PCR was carried out using the profile: 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 1 minute, 72 °C for 60 s and a final extension at 72 °C for 5 minutes. Gel electrophoresis was carried out to analyze the PCR results. Whereas, the gene sequences flanking guide region was amplified using Q5 High-Fidelity 2X Master Mix (New England Biolabs) following the manufacturer's protocol at an annealing temperature of 60 °C. The transgenic lines and WT control PCR product were subjected to next-generation sequencing, CAPS assay, and Sanger Sequencing for mutant identification. Illumina paired-end read amplicon sequencing and Oxford Nanopore Sequencing were performed by CCIB DNA Core (Cambridge, MA, USA) and Plasmidsaurus (Eugene, OR, USA). NGS raw reads were analyzed using the default parameters in CRISPRESSO2 (Clement et al., 2019) and Geneious Prime software. For the CAPS Assay, the PCR products were subjected to overnight *NcoI* restriction digestion at 37°C, followed by migration at 2% agarose gel. Sanger sequencing was also performed to confirm the gene edits. Sanger trace data was also inferred using the CRISPR Synthego ICE v2 tool(Conant et al., 2022).

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

Publication:

1. Mehtab- Singh, Kaye, C., Kaur, R. and Singh, J. (2025) A highly efficient CRISPR-Cas9-based gene-editing system in oat (*Avena sativa L.).Plant Biotechnol. J.,* https://doi.org/10.1111/pbi.70146

2. Mehtab-Singh., Tripathi, RK., Bekele, WA., Tinker, N., Singh, J., (2024). Differential expression and global analysis of miR156/SQUAMOSA promoter binding-like proteins (SPL) module in oat. *Scientific Reports* 14, 9928 https://doi.org/10.1038/s41598-024-60739-7

Meetings and Conference Presentations:

1. Mehtab-Singh., Kaye, C., Kaur, R., Singh, J., (2025). Harnessing CRISPR/Cas9 for Precision Gene Editing in Oats, 6th March, International Symposium on gene Editing, Punjab Agricultural University, Ludhiana, India. (Oral)
2. Mehtab-Singh., Kaye, C., Kaur, R., Singh, J., (2025). Unlocking the CRISPR-Cas9-Based Gene Editing System in Oat (*Avena sativa L.*), Feb 19th , Ottawa Research and Development Centre, Agriculture and Agri-food Canada (AAFC) (Oral)
3. Mehtab-Singh and Singh, J., (2025). CRISPR/Cas9-mediated tweaking of flowering time for oat improvement, Jan 14th , Plant and Microbial Biology Department, University of California, Berkeley. (Invited Talk)

4. Mehtab-Singh., Kaye, C., Kaur, R., Singh, J., (2025). A Highly Efficient CRISPR-Cas9-Based Gene Editing System in Oat (*Avena sativa*), Jan 9-13, Plant and Animal Genomics (PAG 32) meeting, San Diego, USA. (Oral)
5. Mehtab-Singh., Tripathi, RK., Bekele, WA., Tinker, N., Singh, J., (2025). MiR156/SQUAMOSA promoter binding-like proteins (SPL) module and its potential association with floral architecture in oat, Jan 9-13, Plant and Animal Genomics (PAG 32) meeting, San Diego, USA. (Poster)
6. Mehtab-Singh, Bekele, W., Parent, J-S., Nilsen, K., Tinker, N,. Liang, N., Singh, J., (2024). Towards CRISPR/Cas9-Mediated Fine-Tuning of Flowering Time in Oats: A Novel Tool for Plant Breeders, July 21- July 25, American Oat Workers Conference (AOWC), Saskatchewan, Canada. (Oral)
7. Jaswinder Singh, Harnessing Gene Editing for Climate-Resilient Cereals, International Symposium on Harnessing Genome Editing for Climate Resilient Agriculture**,** Punjab Agricultural University, Ludhiana, India (March 3-March 7, 2025) (Invited presentation).
8. Jaswinder Singh, Optimizing Small Grain Cereals Through Gene Editing and Complementary Techniques, The Center for Precision Plant Genomics, 2025 Symposium, University of Minnesota, Minneapolis, USA. May 16, 2025 (Invited presentation).
9. Jaswinder Singh, Gene Editing – New tools for crop improvement, Biotechnology for a Resilient Agriculture, Workshop, USDA workshop, INRA, Rabat, Morocco. September 18, 2024 (Keynote presentation).
10. Jaswinder Singh, Genetic Transformation and Gene Editing for Decoding the Oat Genome, PepsiCo, USA (August 29, 2024) (Invited talk- delivered virtually)

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We are also grateful to our collaborators – Drs. Nicholas Tinker, Wubishet Bekele, Jean-Sébastien Parent from AAFC, Ottawa, and Dr. Kirby Nilsen, AAFC, Branden.

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