Annual Status Report: Research

a) Layman 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.

b) Technical Summary

We hypothesize the role of *VRN3D* in regulating flowering time in oat. However, its transcriptional regulation and association with flowering and yield-related traits remained unravelled. *VRN3D* promoter search revealed the binding sites of SQUAMOSA promoter binding like (SPL) protein, a key regulator of plant reproductive phase transition. Further, we identified 28 *SPL* genes *(AsSPLs)* distributed across all 21 oat chromosomes except for 4C and 6D. Intriguingly, *AsSPL3* showed high transcript abundance during early inflorescence (GS-54) as compared to the vegetative stage (GS-22), indicating its association with reproductive development. Intriguingly, *VRN3* co-expressed with SPL3 at the developing inflorescence stage and further experimentation is required to understand its transcriptional regulation. Hence, various biotin-labelled probes from the *VRN3D* promoter with SPL binding sites were designed, and SPL3 protein was heterologously expressed to validate their interaction via EMSA. To dissect the association of flowering time and yield with *VRN3*, four different types of constructs (pMV3D, pJDMV3D, pJDMV3, and pJDMV3D-Pro) were designed targeting *VRN3D*, *VRN3 (A/C/D)* and *VRN3D* promoter. A total of 405 calli were subjected to particle gun bombardment using different constructs. We have great success in oat genetic transformation with an efficiency of 5.5 % (pMV3D) and 8.1 % (pJDMV3D). The latter construct with the GRF-GIF chimera increased the transformation efficiency by 2.6%. The transgenic lines are regenerating in the greenhouse and will subjected to molecular and phenotypic analysis to identify gene editing events and variation in flowering time. We are also introducing different versions of the *miR172* gene in oat using genetic transformation to play with its floral architecture and flowering time. A total of 891 calli were individually transformed with three different constructs producing 2 successful transgenic events. Regenerated plants will be further screened for more transgenic events and differences in oat panicles.
So far, the project is progressing in line with the predetermined timeline, and barring any unexpected issues, it is anticipated to be successfully completed on schedule, yielding excellent outcomes.

c) **Methods**

Identification of *VRN3D cis-regulatory* elements and *SPL* genes in oat

The 2000bp upstream sequence (promoter region) of the *VRN3D* was searched for *cis*-regulatory elements using the PlantCARE database (Lescot et al., 2002). The GrainGenes (https://wheat.pw.usda.gov/jb?data=/ggds/oat-ot3098v2-pepsico) database was used to obtain the coding, genomic and protein sequences of oat *SPLs* (*AsSPLs*). The barley SBP domain (Pfam: PF03110) was used as a query to perform tBLASTn against the annotated PepsiCo OT3098 Hexaploid Oat v2 pseudomolecules (2021) and the latest Sang genome (Kamal et al., 2022). The SMART tool (http://smart.embl-heidelberg.de/) was used to verify the SBP domain in the SPL protein sequences.

Plant Material, Sample Preparation and RNA Extraction

The oat cultivar Park obtained from PGRC, Saskatoon, Canada was planted in the growth chambers at Macdonald Campus, McGill University. The plants were grown with a 16:8 photoperiod ratio at day and night temperatures of 22°C and 15°C, respectively. A 20:20:20 (nitrogen: phosphorus: potassium) fertilizer was applied after sowing and at the tillering stage to promote plant growth. The young leaf (GS-22), immature panicles (GS-54, GS-75), and mature panicle samples from the oat plants were collected and immediately flash-frozen in liquid nitrogen before storing the samples at -80 °C. Total RNA was extracted according to the manufacturer's protocol using the spectrum plant total RNA kit (Sigma- Aldrich, St. Louis, MO, USA). Following this, RNA was quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA), and gel electrophoresis was performed to check the purity and integrity.

DNAse I Digestion, cDNA Synthesis, and Semi-Quantitative and Quantitative Real-Time PCR (qRT-PCR)

DNA contamination was removed by DNase I treatment of all samples (Promega, USA).. From each sample, 500ng of RNA was taken to synthesize cDNA using the AffinityScript QPCR cDNA Synthesis Kit (Agilent technology, Canada). Due to high sequence similarity amongst the protein sequences of *AsSPLs* homeologs, common primers for each gene homeolog were designed. However, primers specific to *VRN3D* were designed for the co-expression analysis. Optical strip tubes were used to perform qRT-PCR analysis using the Mx3000 qPCR system (Stratagene, USA). Internal controls included the expression of a reference gene *EFIA*, which was recommended as the most consistent housekeeping gene amongst different growth stages in oat (Yang et al., 2020). Relative gene expression was determined using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). For Semi-quantitative PCR Promega Green Master Mix (PCR) was used followed by Gel electrophoresis for analysis.

Protein Vector construction and biotin-labelled probe design

The pET 32a (+) vector was created for heterologous expression of SBP-DNA binding domain sequence of AsSPL3. The sequence was codon optimized and cloned into BamHI- and XhoI-digested pET 32a (+) vector by Genescript (Piscataway,USA), which included translational His tag at N- and C-terminal. DNA probes from sites containing the 'GTAC' core motif were designed from 2000 bp upstream 5' UTR of the *VRN3D* gene. A total of six 5' end biotin-labelled probes were designed from IDT (Integrated DNA technologies, Canada).

Protein expression, purification and quantification.

The AsSPL3 pET 32a (+) protein expression vector was transformed into E. coli rosetta competent cells using the heat shock treatment. Transformed bacteria were grown to obtain primary cell culture using LB media containing kanamycin for selection at 37°C by shaking at 245 rpm overnight. 1mM IPTG was added for recombinant protein expression at 30 °C overnight (Kadoll et al., 2022). His tagged recombinant protein was purified using Ni-NTA fast start kit (QIAGEN) as per the manufacturer's instruction. The recombinant protein was quantified using the Bradford assay (Bradford, 1976).

Guide RNA and construct design

The Geneious software was used to design guide RNA (gRNA) using the Park oat genome available at GrainGenes (https://wheat.pw.usda.gov/jb?data=/ggds/oat-ot3098v2-pepsico) database. Multiple gRNAs were designed using different strategies: specific to VRN3D, common to all the VRN3 copies and targeting various regions of VRN3 promoter. The appropriate guide RNA was selected based on position, off-target score (Hsu et al., 2013) and efficiency score (Doench et al., 2014). Various constructs with different promoters driving the gRNA and Cas9 were designed to test the transformation system. Firstly, the guides were cloned in the previously designed shuttle vector and then ligated to the recipient vector (Kumar et al., 2018) using Golden Gate cloning. Secondly, the JD633 backbone with the GRF-GIF system was used to develop constructs targeting *VRN3* gene (Debernardi et al., 2020). In collaboration with Dr. Parent (AAFC), improved JD633 was designed, containing the oat U6 promoter for driving gRNAs. Three different constructs (pH7WG-miR172-7(WT), pH7WG-miR172-7, and pH7WG-miR172-8) containing different versions of the *miR172* gene were also designed. DH5α cells were used to transform the final constructs and then grown on selective media (Lysogeny broth (LB) supplemented with specific antibiotic). Three QIAGEN Plasmid Midiprep Kit was used to perform plasmid isolation after picking the positive colonies.

Production of Callus

Mature seeds of the oat spring variety Park were used due to their success in particle gun bombardment (Cho et al. 1999). After sterilizing the seeds in 1.5% bleach for 10 minutes, they were put on regeneration media for germination. Upon germination, they were placed on C' media after trimming the grown shoot/roots (Appendix Table 2). For the maintenance of undifferentiated callus, roots and shoots were cut every couple of weeks.

Particle gun bombardment

Before bombardment, a 3 cm diameter circle of callus is initially put on osmotic media for three hours (c' supplemented with 0.2 M mannitol and 0.2 M sorbitol). 6µl of suspended gold stock solution was utilized per plate (60mg of 0.6 µm gold particles in 1ml of 100% ethanol). The supernatant was discarded after centrifuging the suspension for 1 minute at 13,000 rpm. The pellet was again centrifuged after adding 200-300 µl of filter sterile water (FSH2O) and the supernatant was discarded. Again, the pellet was obtained and 6 µg of plasmid DNA was added. Following this, 250 µl of FSH2O from the volume added in DNA, 250 µl of calcium chloride (CaCl2) and 50 µl of spermidine is added. This mixture was briefly vortexed and incubated on ice for 30 minutes. Then the mixture was again centrifuged at 13,000 rpm for 1-2 minutes and 200 µl of ethanol was added after removing the supernatant. Finally, 36 µl of ethanol was added to the pellet to make the plasmids good for bombardment. The microprojectile bombardment was accomplished using the BioRad PDS-1000/He system. A total of 405 callus pieces were separately bombarded with

various constructs. The next day they were transferred to C' media for a week. Three rounds of selections were performed by placing the bombarded callus on C' media supplemented with 20 mg/L of Hygromycin. The healthy calli were placed on the same media and the process was continued for a few weeks. The selected calli were allowed to regenerate, producing roots and shoots on the regeneration media. Later, they were transferred to pots in the greenhouse. Furthermore, 891 calli were transformed with three different constructs containing different versions of the *miR172* gene and were subjected to similar aforementioned conditions.

Genomic DNA extraction and analysis of transgenic plants

The genomic DNA was extracted from the regenerating lines. A thermocycler and Promega Green Master Mix (PCR) were used to amplify the incorporated construct by *Hygromycin* gene primers. The PCR product was subjected to gel electrophoresis on 1% agarose gel with the wild type (v. Park) and water as a negative control, and the construct as a positive control.

d) Progress:

Objective 1:

Identifying regulatory sequences in *VRN3* (7D) gene and refining gene editing in oat.

Oat SPL3 as a putative regulator of *VRN3D*

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). *OsSPL14* regulates the branching of panicles and increases yield in rice, whilst another SPL gene, named *tasselsheath4*, promotes bract development and meristem initiation in maize (Luo et al., 2012).

Since *VRN3D* is also a flowering gene, we hypothesize 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) (Table 1). Biotin-labelled probes were designed for DNA-protein interaction analysis covering these binding sites (Appendix Table 1).

Table 1. *Cis-regulatory* elements in the promoter of *VRN3D*.

Factor or Site Name	Loc.(Str.)	Signal Sequence
ROOTMOTIFTAPOX1	14 (+)	ATATT
CAATBOX1	16 (-)	CAAT
ARR1AT	18 (+)	NGATT
GT1CONSENSUS	21 (-)	GRWAAW
GT1GMSCAM4	21 (-)	GAAAAA
LTRE1HVBLT49	23 (-)	CCGAAA

TATABOX3	95 (-)	TATTAAT
POLASIG1	109 (-)	AATAAA
CAATBOX1	112 (-)	CAAT
CCAATBOX1	147 (-)	CCAAT
SORLIP2AT	163 (+)	GGGCC
WRKY71OS	191 (-)	TGAC
GTGANTG10	192 (-)	GTGA
CACGTGMOTIF	193 (-)	CACGTG
WRKY71OS	225 (+)	TGAC
AMYBOX1	276 (+)	TAACARA
GTGANTG10	290 (-)	GTGA
ASF1MOTIFCAMV	357 (-)	TGACG
CBFHV	405 (+)	RYCGAC
GCCCORE	427 (+)	GCCGCC
SBP-SPL	479 (+)	CCGTACA
GATABOX	548 (-)	GATA
SORLIP2AT	601 (+)	GGGCC
GCCCORE	695 (+)	GCCGCC
SBP-SPL	756 (+)	GTAC
SBP-SPL	1215 (+)	GTAC
SBP-SPL	1562 (+)	GTAC
SBP-SPL	1605 (+)	GTAC
SBP-SPL	1621 (+)	GTAC
SBP-SPL	1671 (+)	GTAC
SBP-SPL	1688 (+)	GTAC

To identify a specific SPL regulating *VRN3*, a genome-wide search was carried out in the oat genome. It reported 28 oat *SPLs* (*AsSPLs)* distributed across all 21 oat chromosomes except for 4C and 6D. Twenty-five *SPL*s were identified in the latest *Avena sativa cv. Sang* genome V1 and three additional *SPLs* (*AsSPL1D, AsSPL6D, AsSPL17D*) were identified in the *Avena sativa cv. OT3098 V2* genome. As expected, all *AsSPLs* had three copies in the genome, except for *AsSPL23,* which had a single copy in the D genome. Interestingly, *AsSPL3s, AsSPL6s, AsSPL11s*, and *AsSPL15s* were not situated on homoeologous chromosomes, likely due to ancestral chromosome rearrangements. The number of exons ranged from two to 11, whilst the deduced proteins ranged in length from 179 to 1114 (Table 2).

Table 2. Characterization of identified *SPL* genes in *Avena sativa*

Gene[a]	Gene Symbol[b]	CDS length[c]	Domain[d]	Deduced Protein (aa)[e]	Ch[f]	Genomic Position[g]	Exon[h]
AsSPL1A	AVESA.00010b.r2.3AG0429080	2130	SBP	710	3A	150194572-150199444	11
AsSPL1C	AVESA.00010b.r2.3CG0485440	2592	SBP	864	3C	351718169-351724905	11

*AsSPL1D**	AVESA.00001b.r3.3Dg0000777	2643	SBP	881	3D	128038037-128043795	11
AsSPL3A	AVESA.00010b.r2.1AG0030000	1659	SBP	553	1A	393533069-393538332	5
AsSPL3C	AVESA.00010b.r2.6CG1116620	1149	SBP	383	6C	142489501-142495376	3
AsSPL3D	AVESA.00010b.r2.2DG0387650	1647	SBP	549	2D	68159213-68163589	5
AsSPL6A	AVESA.00010b.r2.4AG0617780	2082	SBP	694	4A	321184138-321188500	9
AsSPL6C	AVESA.00010b.r2.7CG0713010	2238	SBP	746	7C	13544283-13550860	10
*AsSPL6D**	AVESA.00001b.r3.4Dg0002398	2871	SBP	956	4D	342088718-342095542	11
AsSPL9A	AVESA.00010b.r2.1AG0053930	2553	SBP	851	1A	294768922-294779215	10
AsSPL9C	AVESA.00010b.r2.1CG0079470	2697	SBP	899	1C	418196430-418208251	11
AsSPL9D	AVESA.00010b.r2.1DG0172650	2550	SBP	850	1D	276378031-276388313	10
AsSPL11A	AVESA.00010b.r2.6AG1006610	735	SBP	245	6A	1679912-1683980	3
AsSPL11C	AVESA.00010b.r2.6CG1110970	1005	SBP	335	6C	180579284-180582815	4
AsSPL11D	AVESA.00010b.r2.2DG0382760	1005	SBP	335	2D	90934967-90938970	4
AsSPL13A	AVESA.00010b.r2.2AG0198950	537	SBP	179	2A	24988625-24991456	2
AsSPL13C	AVESA.00010b.r2.2CG0276270	546	SBP	182	2C	113571449-113574550	2
AsSPL13D	AVESA.00010b.r2.2DG0349760	537	SBP	179	2D	236690645-236693452	2
AsSPL15A	AVESA.00010b.r2.7AG1218390	3318	SBP	1106	7A	134554545-134560310	10
AsSPL15C	AVESA.00010b.r2.5CG0931600	3342	SBP	1114	5C	15625436-15630899	10
AsSPL15D	AVESA.00010b.r2.7DG1390040	3318	SBP	1106	7D	49217131-49222850	10
AsSPL16A	AVESA.00010b.r2.5AG0853240	1317	SBP	439	5A	448045544-448052002	3
AsSPL16C	AVESA.00010b.r2.5CG0880720	1299	SBP	433	5C	507845009-507850886	3
AsSPL16D	AVESA.00010b.r2.5DG0956210	1329	SBP	443	5D	401441491-401447497	3

AsSPL17A	AVESA.00010b.r2.5 AG0851230	1167	SBP	389	5A	441621396-441625091	3
AsSPL17C	AVESA.00010b.r2.5 CG0882850	1179	SBP	393	5C	499731050-499735457	3
AsSPL17D *	AVESA.00001b.r3.5 Dg0002304	1155	SBP	384	5D	399247804-399251340	3
AsSPL23D	AVESA.00010b.r2.4 DG0772040	1188	SBP	396	4D	337532631-337537680	3

. [a]Nomenclature of oat *SPLs* in this study. [b]Gene accession number in the oat database. [c]Length of coding sequence. [d]Domain prediction by SMART tool. [e]Number of amino acids in the protein sequence. [f]Chromosomal location of *AsSPL* genes. [f]Chromosomal coordinates of *AsSPL* genes in the oat genome. [h]Number of exons in *AsSPL* genes. [i]Predicted subcellular location of *AsSPL* genes. **AsSPLs* identified in the *Avena sativa cv. OT3098 V2* genome.

To further explore the specific SPL involved in flowering transition, co-expression analysis was performed at different growth stages involving young leaf (GS-22), immature panicles (GS-54, GS-75), and mature panicle. A wide range of differential expression patterns were observed in the selected *AsSPLs* in different tissues (Fig. 1). A relatively high expression of *AsSPL9s* was observed at the vegetative stage, i.e., tillering (GS-22). On the contrary, *AsSPL1s, AsSPL3s, and AsSPL15s* showed lower expression in the vegetative stage (GS-22) but higher in the developing inflorescence stage (GS-54). Intriguingly, *AsSPL3s* had an extremely high expression in the inflorescence emergence stage, implying their putative role in the vegetative to reproductive phase change in oat. The expression profiling was consistent in *AsSPL6s* and *AsSPL11s* across the different growth stages. Most of the selected *AsSPLs* showed comparatively low expression in the mature spikes. These results make SPL3 an excellent candidate for gene editing in flowering time studies. Semi-quantitative PCR showed transcripts of *VRN3 (A/C/D)* at the tillering and inflorescence emergence stage, while the *VRN3D* transcripts were only seen during the tillering stage (Figure 2). Additional experiments will be carried out to investigate the interaction between SPL3 and *VRN3* in oat.

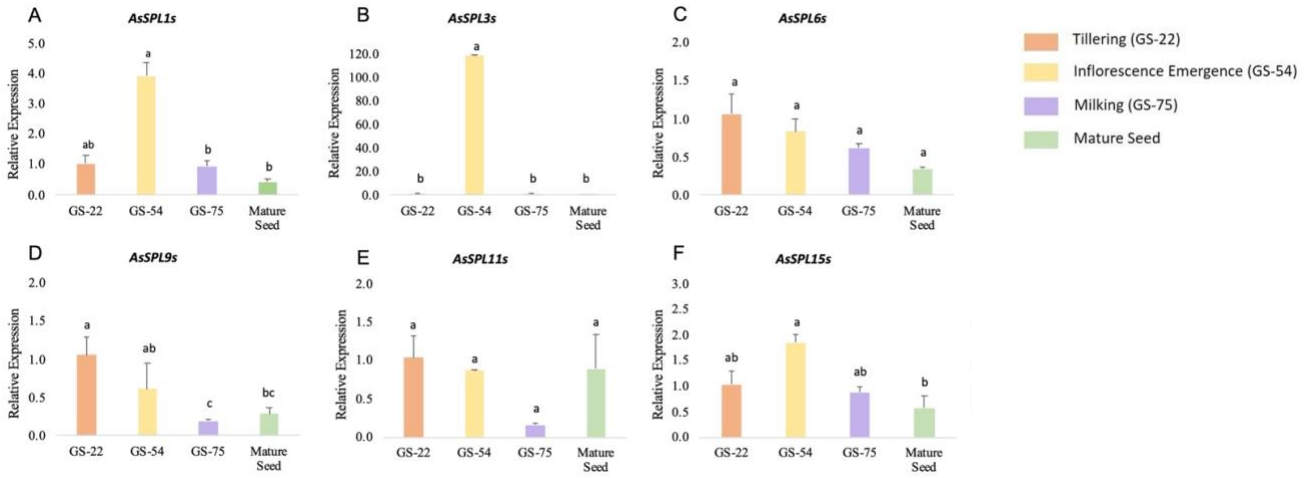


Figure 1. Expression of *AsSPL* genes during growth phase transition in oat. Error bars are indicated. The lowercase letter above the bar indicates the significant difference ($P \leq 0.05$) among the different growth stages.

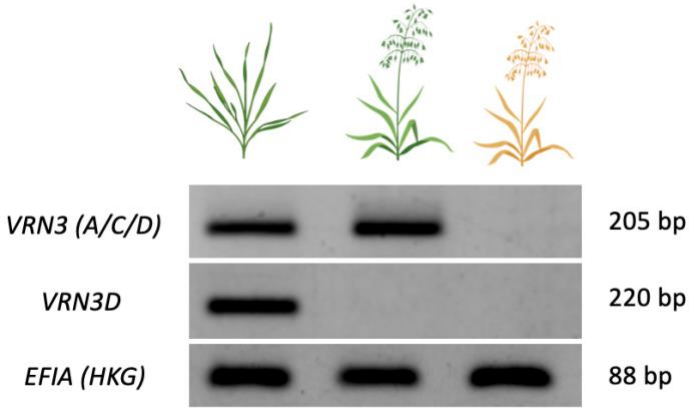


Figure 2. Semi-quantitative PCR of *VRN3 (A/C/D)* and *VRN3D* at different oat growth stages.

Heterologous expression of AsSPL3

The DNA binding SBP domain of SPL3 was expressed in E. coli rosetta cells. The expressed protein was purified and confirmed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Figure 3). The protein size of 27.25 kDa can be seen on the gel in the Elute 1 and 2 lanes. Bradford's assay quantified 1.2 mg/ml purified protein which will be used to confirm the binding of AsSPL3 on *VRN3D*'s promoter using Electrophoretic Mobility Shift Assay (EMSA).

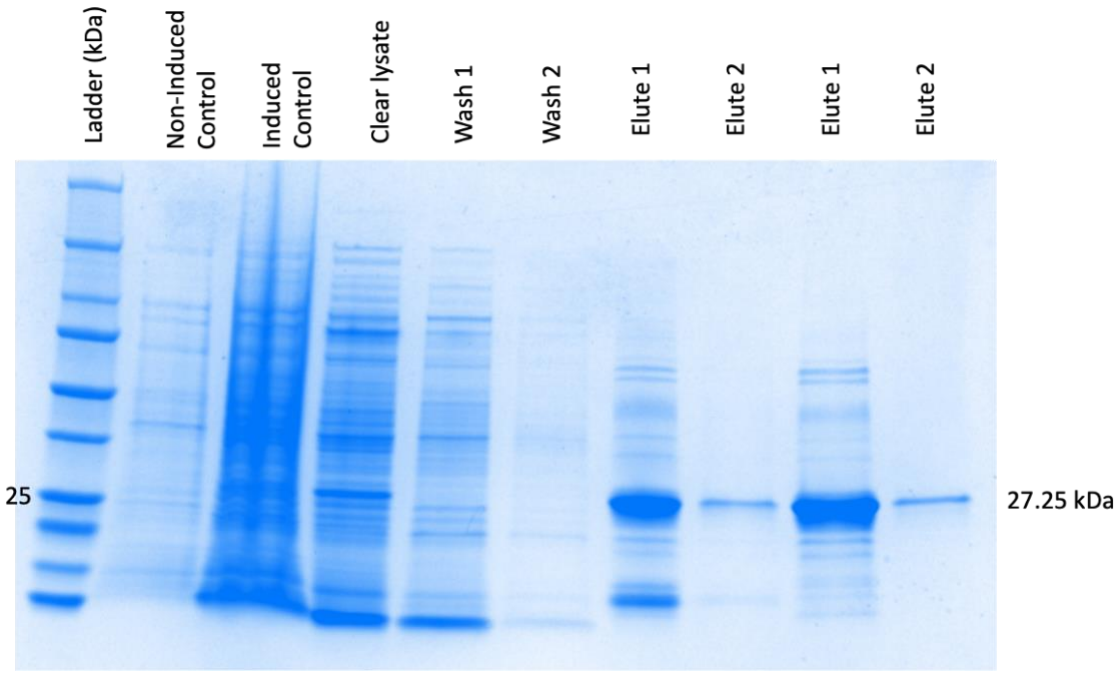
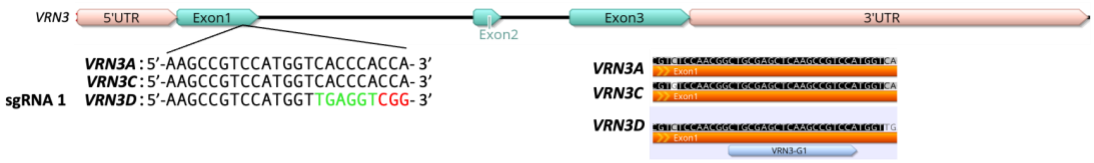


Figure 3. SDS-PAGE of AsSPL 3 protein

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

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 4). 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 3). All the calli regenerated into plants but produced no edits. pJDMV3D and pJDMV3 contain guides specific to *VRN3D* and *VRN3 (A/C/D)*, respectively. They have a JD633 backbone with TaU6 promoter driving the guides, along with morphogenic GRF-GIF chimera. The transformation of 75 calli with pJDMV3 yielded no transgenic events. In another experiment, we bombarded 135 calli with pJDMV3D and successfully generated 11 transgenic lines (TE= 8.1%) on *Hyg* selection (20mg/L) confirmed through PCR (Figure 4 and Figure 5). The use of JD633 with GRF-GIF chimera increased the transformation efficiency by 2.6%, parallel to findings reported in other crops (Biswal et al., 2023). The plants are regenerating in the greenhouse and will subjected to deep and sanger sequencing to find the gene editing events at the *VRN3* locus. 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 6). 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.



S.No.	Construct Name	Target	Guide	Information	Progress	Method
1	pMV3D	*VRN3D*	AAGCCGTCCATGGTTGAGGT	Guide is driven by HvU3 promoter	Transformed	Biolistic
2	pJDMV3D	*VRN3D*	AAGCCGTCCATGGTTGAGGT	TaU6 promoter, JD633 with GRF/GIF chimera	Transformed	Biolistic *Agrobacterium*
3	pJDMV3	*VRN3(A/C/D)*	CTGCGAGCTCAAGCCGTCCA	TaU6 promoter, JD633 with GRF/GIF chimera	Transformed	Biolistic
4	pJMV3D-Pro	*VRN3D* Promoter	8 guides	AsU3 promoter, JD633 with GRF/GIF chimera	Transformed	Biolistic

Figure 3. gRNA and construct design for gene editing *VRN3* in oat.

Table 3. Summary of *VRN3* transformations in oat.

S.No.	Construct	No. of cali bombarded	Transgenic cali	Transformation Efficiency (TE) (%)	Regeneration Efficiency (%)
1	pMV3D	90	5	5.5	100
2	pJDMV3	75	0	0	NA
3	pJDMV3D	135	11	8.1	100
4	pJDMV3D-Pro	105	TBD	TBD	TBD

NA- Not applicable; TBD- To be declared.

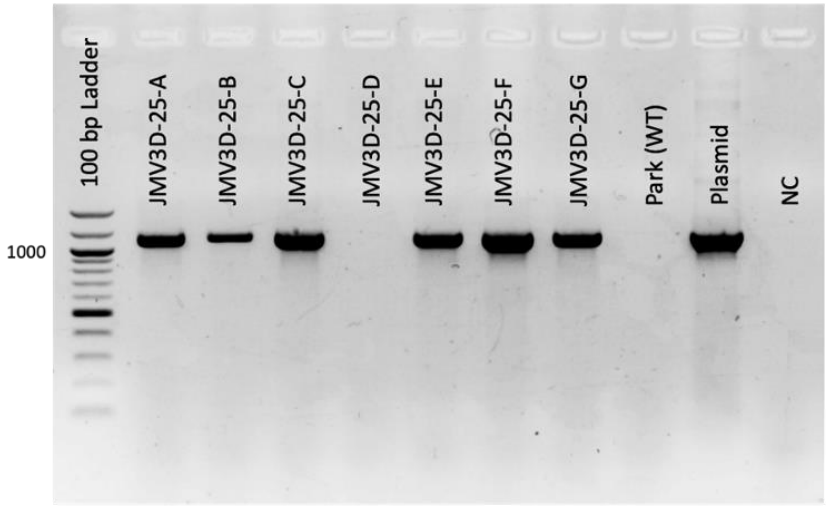


Figure 4. Successful transformants in oat confirmed using *Hyg* PCR.

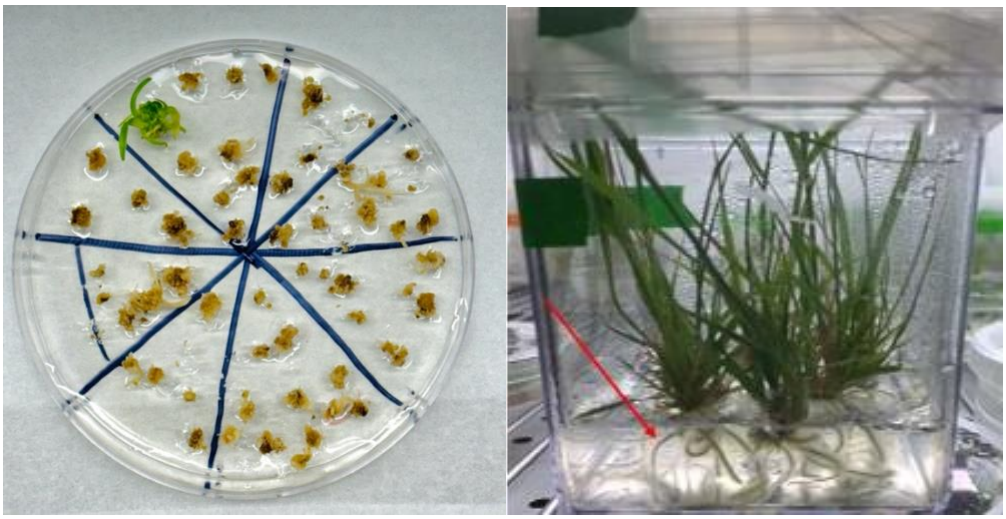


Figure 5. Transgenic calli selected on *Hygromycin* (Left); Transgenic plants rooting in the rooting media (Right)

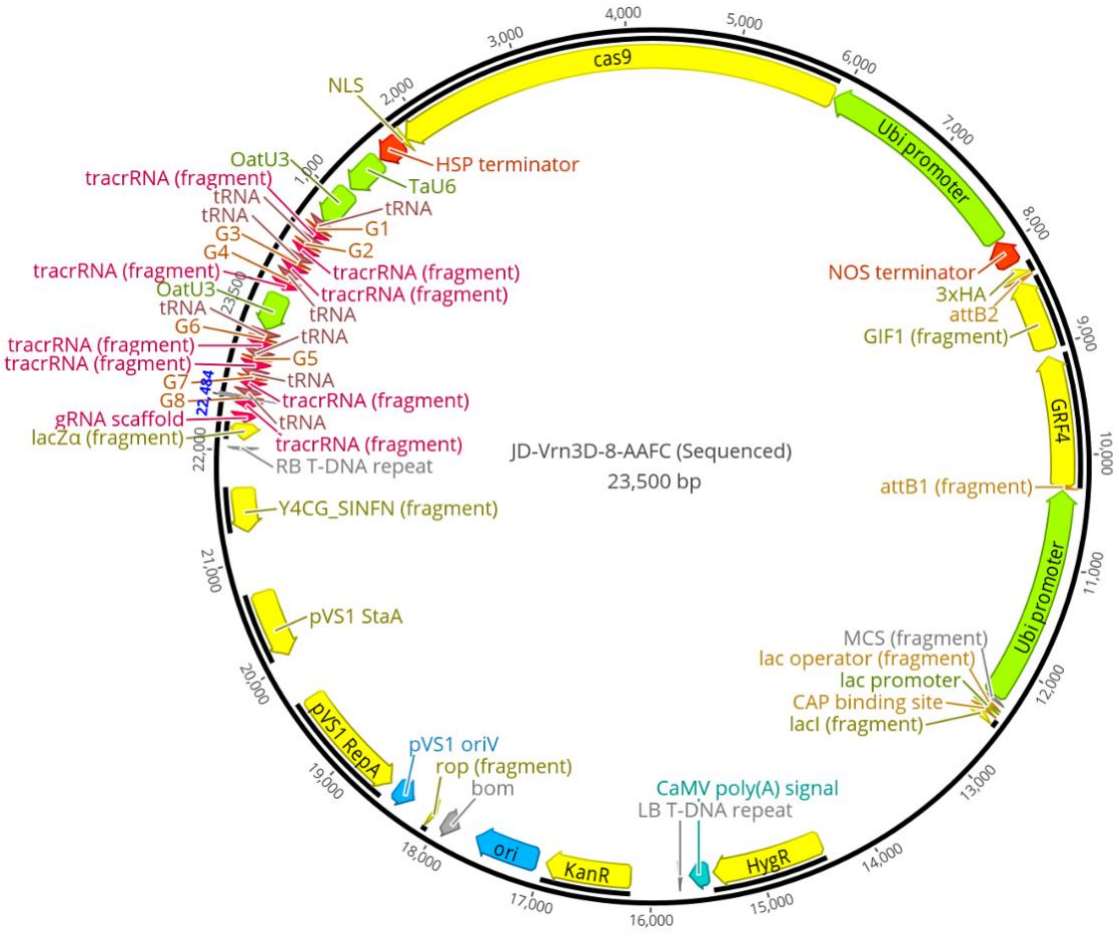


Figure 6. pJDMV3D-Pro construct map with improved oat U3 promoter driving 8 gRNAs.

miR172 precursors are also upregulated by SPLs to facilitate vegetative to reproductive transition in plants (Wang and Wang, 2015). Three different constructs that contained different versions of the *miR172* gene were used for particle bombardment. In the initial three bombardments, about 245 calli were bombarded with pH7WG-miR172-7(WT) construct, 210 calli were bombarded with pH7WG-miR172-7mu construct, while 105 calli were bombarded with pH7WG-miR172-8 construct (Table 4). Collectively for these constructs, a total of 45 plants were regenerated. DNA was extracted from these plants and PCR screening was carried out with miR172insert_end () & JNOSR () primers. Plant 13 and 22 were found positive during the PCR (Fig. 7). Plant-13 was transformed with pH7WG-miR172-7(WT) construct and plant-22 was transformed with pH7WG-miR172-7mu construct.

Table 4: Summary of bombarded calli and regenerated plants

Construct	Number of calli bombarded	Number of calli during regeneration	Number of plants regenerated
pH7WG-miR172-7(WT)	245	100	30
pH7WG-miR172-7mu	210	55	11
pH7WG-miR172-8	105	14	0

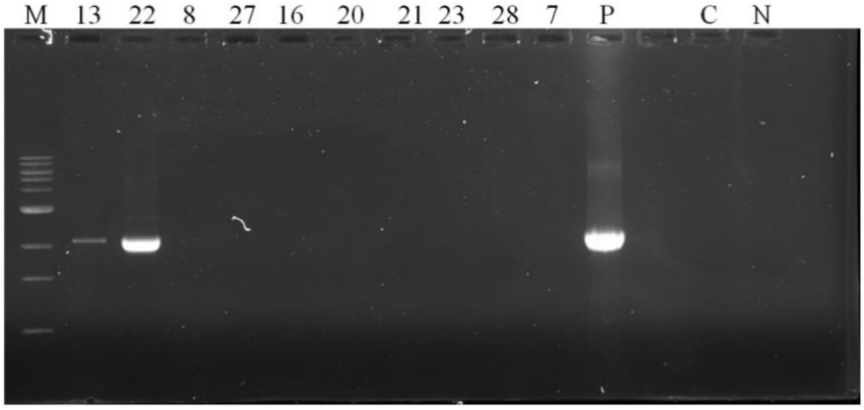


Fig. 7. PCR screening of regenerated plants with miR172insert_end & JNOSR primers. P- positive control (pH7WG-miR172-7(WT) plasmid); C- WT control (Oat (Park)); N- No template control; M- 1 kb DNA ladder (NEB).

Recently, another bombardment experiment was carried out with all three constructs during which 99 calli were bombarded with pH7WG-miR172-7(WT) construct, 123 calli were bombarded with pH7WG-miR172-7mu construct and 109 calli were bombarded with pH7WG-miR172-8 construct (Table 5). These calli are currently undergoing selection with hygromycin and the hygromycin-resistant calli will be transferred to regeneration media for regeneration of plants.

Table 5: Summary of fourth bombardment

Construct	Number of calli bombarded	Number of calli undergoing selection
pH7WG-miR172-7(WT)	99	91
pH7WG-miR172-7mu	123	104
pH7WG-miR172-8	109	106

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

Publication:

1. 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. Jaswinder Singh (2024) Accelerating Genetic Enhancement in Small Grain Cereals Through Technological Innovations, National Association of Plant Breeders, USA (April 26, 2024) (Invited Webinar)
2. Jaswinder Singh (2024) Gene Editing – New and optional tools to integrate into breeding programs, Prairie Grain Development Committee Annual Meeting, Saskatoon, Saskatchewan, Canada (February 28, 2024) (Plenary lecture).
3. Jaswinder Singh (2023) Technological advances to expedite genetic improvement in small grain cereals, 5th Canadian Wheat Symposium (CWS) 2023, Vancouver, British Columbia, November 13-16, 2023) (Keynote presentation).
4. Jaswinder Singh (2023) Decoding and Tuning of Genes in Small Grain Cereals, CSIRO Plant Industry, Canberra, Australia (Sept 25, 2023).
5. Jaswinder Singh (2023) Genetic Transformation and Decoding the Oat Genome, "Precise Genomics and Genome Editing for Food Security and Environmental Sustainability", Plant and Animal Genomics meeting, Perth, Australia (Sept 20, 2023).
6. Jaswinder Singh (2023) Genetic Transformation and Gene Editing for Decoding the Oat Genome, Natural Resources Institute Finland (Luke), Genomics & Breeding (GEJA), Finland (August 29, 2023) (Virtual presentation).
7. Jaswinder Singh (2023) Tuning and tweaking of genes in small grain cereals to ameliorate their genetics, University of British Columbia, Vancouver, Canada (June 29, 2023).
8. Mehtab-Singh (2024) Harnessing gene editing to develop climate responsive oats, John Abbott College, Montreal, Canada (April 19, 2024) (Oral).
9. Mehtab-Singh (2024) Making Oats Punctual. Lister Family Engaged Science 3-Minute Thesis Competition, McGill University, Montreal, Canada (Nov 29, 2024) (Oral).

10. Mehtab-Singh (2023) MiR156/SQUAMOSA promoter binding-like proteins (SPL) module mediated vegetative to reproductive transition in oat. Canadian Society of Plant Biologists, Eastern Region Meeting, Nov.31-Dec1, Montreal, Canada. (Poster)

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g) **Appendices**

Table 1. Primers and probes used in the aforementioned objectives:

S.No.	Primer Name	Sequence (5'- 3')
1	q-AsSPL1-F	GCGAAGAAGAGAGACCTGCAA
2	q-AsSPL1-R	ACACCTTGTGCCTCTTGTGG
3	q-AsSPL3-F	CTTCAGGATGCTCTCTCTCT
4	q-AsSPL3-R	GTGTGCATAGCAGGGTTG
5	q-AsSPL6-F	GAACTGGGCTGGCTTCTTCA
6	q-AsSPL6-R	TTTGGTCACAGCACACCACT
7	q-AsSPL9-F	CCTGAGTTGGACGAGATGGC
8	q-AsSPL9-R	AGCCTGGTACTTGGCACTTC
9	q-AsSPL11-F	AAGCATCTTGCCTCGTCCAG
10	q-AsSPL11-R	TGCCATCCAAGGTAAAACGGA
11	q-AsSPL15-F	GCTCGCCCCTACATCCATTC
12	q-AsSPL15-R	ATCCAGCCTCTCCCACTTGA
13	q-AsEFIA-F	GTGAAGATGATTCCCACCAAGC
14	q-AsEFIA-R	CCTCATGTCACGCACAGCAAA
15	DeepSeq-AsVrn3D-F	TCGCTGTTCCAGGCAGCATA
16	DeepSeq-AsVrn3-R	CGAGTGTGTAGAAGGTCCTCATC
17	pRGEB32_7045F	TGCTCAACACATGAGCGAAACC
18	pRGEB32_8155R	TGAACTCACCGCGACGTCTGTC
19	VRN3D-Pi1	TCTCTCCGGCCCGTACATCCTATCTGAATG
20	VRN3D-Pi2	TGATTTTTCGGCGGTACTTTATTTGATGGT
21	VRN3D-Pi3	CTCATCATATGATGTACTTGTTTTCCTTCA
22	VRN3D-Pi4	AATTAACTGTGGAGTACCACAAATCTACAC
23	VRN3D-Pi5	CCTGCACCGTAACAAGTACGGGCATCGAGTACCACCACATGCAGC
24	VRN3D-Pi6	ATCTCTTAGTAAGGTGTACTATGAGCTGGTACTTCCCTCTGTCGA

Table 2. Components of regeneration, rooting and C' Media.

Components	Regeneration	Rooting	C'

MS salts (g/L)	4.4	4.4	4.4
Maltose (g/L)	--	--	30
Sucrose (g/L)	30	30	--
Casein hydrolysate (g/L)	--	--	1
Proline (g/L)	--	--	0.69
Myo-inositol (g/L)	--	--	0.25
Thiamine HCl (mg/L)	1	1	1
Pyridoxine HCl (mg/L)	0.5	0.5	--
Nicotinic acid (mg/L)	0.5	0.5	--
CuSO4	0.16 mg/L	0.16 mg/L	5 μM
2,4-D (mg/L)		2	
BAP (mg/L)	1	--	0.5

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