Genomic and Phenotypic Characterization of Early Flowering (Epi)Mutants Derived from 5-Azacytidine Treated Flax (*Linum usitatissimum* L.) Cultivar 'Royal'

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INTRODUCTION

Canada is the world leader in flax production and exports. In order to expand the area of flax production beyond current growing areas typically restricted to southern Saskatchewan and Manitoba, into northern parts of the prairies, there is a need to develop early maturing cultivars to escape the potential frost damage during the physiological seed maturity phase (Figure 1).

Flowering time is a complex trait governed by nearly 300 genes associated with eight different physiological pathways. These include responses to photoperiod, aging, vernalization, ambient temperature and regulations through the circadian clock, phytohormones, sugars and the autonomous flowering pathways (Bouché et al. 2016). Hence, there is a need to understand the molecular genetic basis of flowering time for developing early flowering, and consequently early maturing lines.

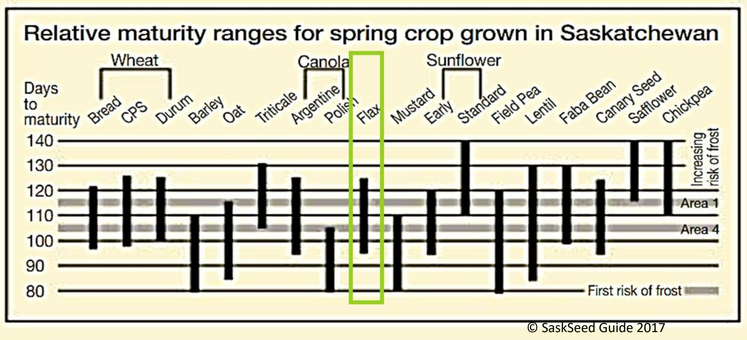

Figure 1. Relative maturity range for spring crops grown in Saskatchewan. Flax grown in the northern part of the prairies is susceptible to frost damage earlier in the season than rest of the growing regions (SaskSeed Guide 2017).

MATERIALS

- Three early flowering lines RE1, RE2 and RE3 were derived from an epimutagenized population of the cultivar 'Royal' with 5-azacytidine (Fieldes and Amyot 1999; Figure 2). RE2 flowered and matured earlier and was significantly less photoperiod sensitive than its progenitor 'Royal' (Jia Sun 2015).

- Recombinant Inbred Lines (RILs) from the reciprocal crosses of 'Royal' and 'RE2' were developed at the Crop Development Centre, University of Saskatchewan.

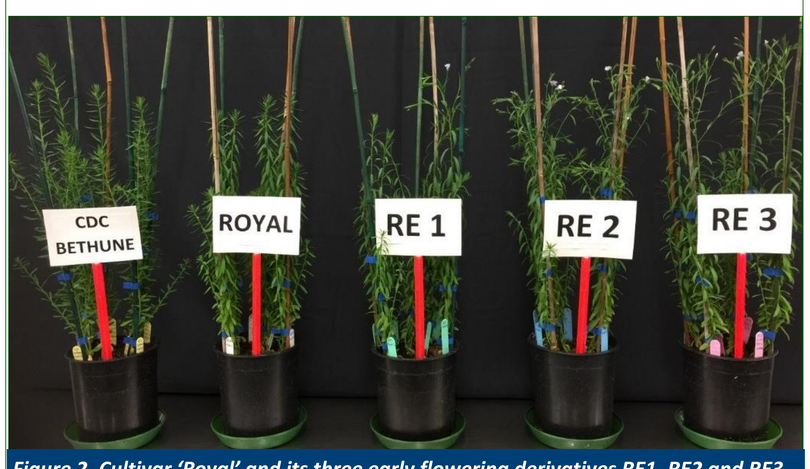

Figure 2. Cultivar 'Royal' and its three early flowering derivatives RE1, RE2 and RE3. (Cabinet grown, under long day- 16 hours light conditions; 31 days after seeding).

- 5-azaC, a DNA methylation inhibitor, is a cytidine analog with nitrogen atom in place of carbon at the fifth position of the ring (Figure 3). 5-azaC treatment results in genome-wide demethylation due to reduction in available DNA methyltransferases by the formation of irreversible complexes, in addition to specific hypomethylation at sites where 5-azaC is incorporated (Pecinka and Liu 2014).

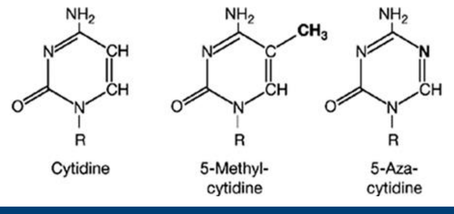

Figure 3. Chemical structure of cytidine and 5-azacytidine (Fenaux, 2005).

METHODS

Figure 4. Early flowering segregants (RIL) derived from the cross 'Royal X RE2' grown at Kernen crop research farm, Saskatoon.

- **Phenotyping in the Field**: The RILs from the crosses Royal x RE2 and RE2 x Royal (Figure 4) were grown in Modified Augmented Design 2 (MAD2; Figure 5) at Kernen crop research farm, and different phenological traits such as start of flowering (5%), end of flowering (95%) were evaluated in 2015 and 2016.

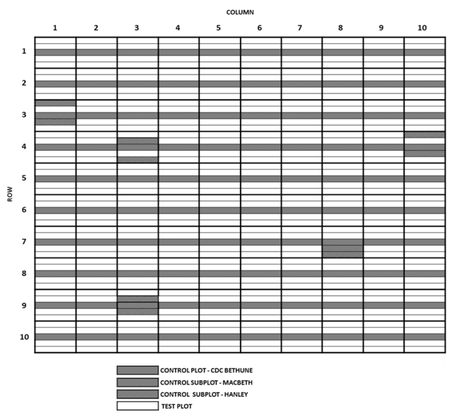

Figure 5. Modified augmented design type 2 (Lin and Poushinsky, 1983): The field plot is divided into whole plots and which in turn is divided into subplots. The main plot control is planted in the middle subplot of each whole plot, and subplot controls are randomly placed in a subset of whole plots.

- **QTL sequencing** (QTL-seq;Takagi et al. 2013): A novel strategy combining the advantages of bulk segregant analysis and next generation sequencing (Figure 6).

  -A mapping population is generated using a biparental cross.

  -The segregating population is evaluated for the trait of interest and two extreme bulks at either end of the distribution are generated based on the phenotypic data.

  -The resequencing data of the extremes is aligned to the parent's reference genome and a statistical parameter called SNP index is calculated using the QTL-seq pipeline.

  -Except for the genomic region responsible for the trait of interest, other regions would be equal from both the parents.

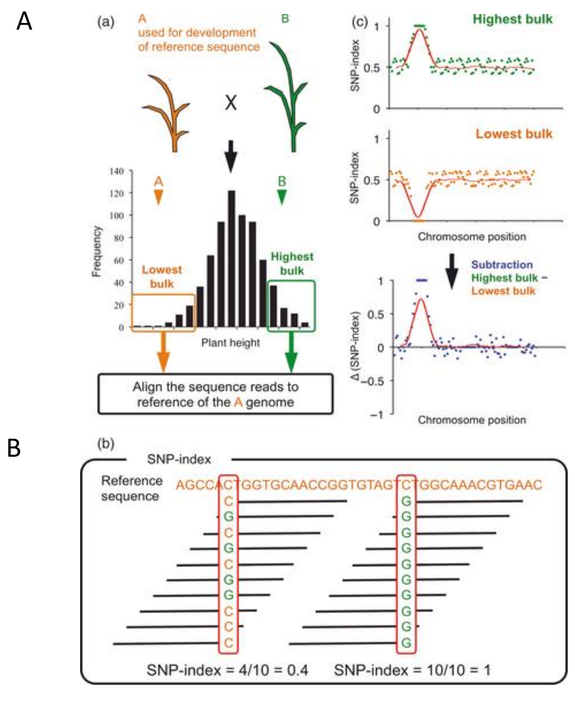

Figure 6. A. Principle of QTL-seq; B. Estimation of SNP-index, which is the ratio between the count of alternate SNPs to total number of reads aligned to the reference assembly corresponding to a given SNP position. If the coverage of a nucleotide position is ten, and if four reads contain a nucleotide different from the reference, the SNP index is 0.4 (Takagi et al. 2013).

- **Whole Genome Bisulfite Sequencing (WGBS):** The differential methylated regions (DMRs) resulting from 5-azaC exposure may underline the trait variation and hence to investigate the potential epigenetic basis of the early flowering trait WGBS will be carried out.

  -The same set of individuals selected for DNA-seq will undergo WGBS to profile molecular variation derived from DNA methylation since the unmethylated cytosine on addition of bisulfite anion undergoes a chemical transformation to uracil and will be read as thymine following PCR and sequencing (Peng et al. 2016).

  -The WGBS data will be analysed using bioinformatic pipelines to interpret SNPs polymorphisms based on methylation status (epigenetic) rather than true genetic SNP variants.

RESULTS OBTAINED SO FAR

The values of the segregating RIL population for different phenological traits and their comparison between years with parents and checks is given below (Table 1) and their frequency distribution is given in the Figure 7.

Table 1. Mean values of the parents, checks and RIL population for phenological traits in the years 2015 and 2016: Early season vigour: rating taken when CDC Bethune is 10-15 cm tall; scale of 1 to 9 (1=extremely weak, 5= average vigour (e.g., CDC Bethune), and 9=extremely vigourous). Stem/straw dry down: rating taken at the same time as maturity rating; scale of 1 to 9 (1 = grass green stems, 3 = green stems, 5 = pale green stems, 7 = yellow stems, 9 = brown stems); all stems within a hill hardly ever had the same stem colour so averages were taken, e.g., if a hill had 50% green stems and 50% pale green stems, then a score of 4 was given.

Phenological traits	Royal		RE2		Royal x RE2 and RE2 X Royal RILs		CDC Bethune		CDC Sorrel	
	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
Early vigour (1-9)	8.6	4.5	6.2	6.0	6.7 (4 - 9)	5.2 (1 - 9)	8.2	4.0	8.1	6.0
Start of flowering (5%)	51.0	37.4	47.0	30.9	50.6 (47.0 - 64.0)	37.0 (29.6-52.1)	53.0	43.8	51.0	41.1
End of flowering (95%)	59.0	44.7	56.0	38.3	58.5 (52.0 - 73.0)	44.2 (37.1 - 59.3)	62.0	50.7	60.0	49.8
Days to maturity	54.0	59.0	35.0	64.5	51.3 (28.0 - 63.0)	57.2 (19.6 - 78.9)	56.0	78.9	65.0	81.2
Stem dry down (1-9)	5.5	8.1	8.1	9.0	4.1 (1 - 9)	8.1 (3 - 9)	4.1	5.0	4.9	5.0
Height (cm)	99.0	98.0	97.0	88.0	99 (92.0 - 110.0)	98 (90.0 - 108.0)	98.0	106.0	106.0	101.0

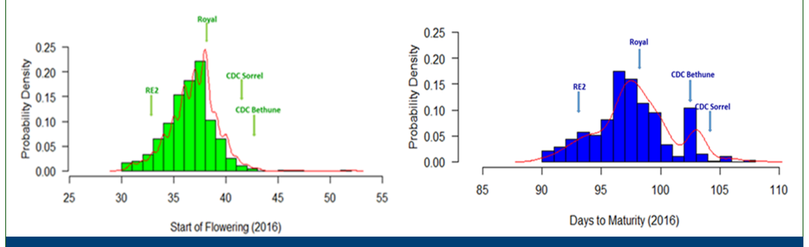

Figure 7. Kernel density plot for start of flowering and days to maturity in the year 2016

POTENTIAL PROJECT OUTCOMES

- Identification of intervals in the flax genetic map with SNP/genomic rearrangements associated with the flowering time variation which can be used to develop diagnostic DNA markers useful for marker-assisted selection of early flowering genotypes.

- Identification of possible epigenetic basis of the early flowering trait, first of its kind in a crop genome.

- Knowledge generated regarding the candidate genes harboring the genomic regions associated with early flowering will be transferable to other prairie crops by identifying 'orthologous' candidate genes.

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