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Frequency of vernalization requirement associated dominant *VRN-A1* gene and earliness related *Esp-A1* candidate genes in advanced wheat mutant lines and effect of allele on flowering time

Abstract: Seeds of the spring bread wheat variety Zhenis, registered in Kazakhstan, were irradiated with 100 and 200 Gray doses from a [^{60}Co] γ-ray radiation source. Promising advanced M_5 mutant lines were obtained and evaluated for mapping density of vernalization requirement associated dominant VRN-A1 gene and earliness related Esp-A1 candidate genes in 30 spring wheat mutant lines along with parent. Differences among generated mutant lines and parent were observed on frequency of distribution of VRN-A1 gene and Eps-A1 candidate gene alleles. Allelic variation at the VRN-A1 loci was observed in the 8 mutant lines (26.6%), mostly in 100 Gy mutant lines. A dominant (VRN) allele at its loci, which was present and indicated in these lines is sufficient to confer a spring growth habit. As for the candidate gene Esp-A1, eleven 100 Gy M_5 mutant and six 200 Gy M_5 lines, had Esp-A1 candidate gene allele. The two lines 24(2) and 43(4) carrying dominant allele of VRN-A1 had higher thousand kernel weight. Mutant lines carrying allele of Eps-A1 candidate gene found significantly differed from the parent especially in 100 Gy lines (73.3%) in flowering time.

Key words. spring wheat, mutation breeding, M_5 mutant lines, VRN-A1 allelic variation, Eps-A^{m}1 candidate gene allele.

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the staple crops with global economic importance and it plays an imperative role in food security. Wheat is successfully grown in different geographical regions of the world from 67°N to 45°S [1]. Optimum growth temperature for wheat is 25°C, but satisfactory yields have been achieved in temperatures ranging from 3 to 32°C [2]. The cultivation of hexaploid wheat in a wide range of environments has become possible mainly due to selection of optimum flowering time [3]. Wheat yield has been increased globally through modification of its developmental pattern that best suit particular growing conditions.

The optimization of flowering time in wheat is critical for plant growth and development in seed crops, to maximize grain yields. Flowering time is a complex character, which exhibits a continuous variation. Pre-anthesis developmental phases in wheat are mainly controlled by vernalization (*Vrn*) and photoperiod (*Ppd*) response, and earliness per se genes [4]. Therefore, adaptability and yield potential of wheat in different agroclimates are mainly determined by these three genes systems and their interactions with growing temperatures [3].

Varietal differences in flowering time other than those due to photoperiod and vernalization response have been observed in wheat [15]. Such genetic differences are termed ‘‘earliness per se’’, ‘narrow-sense earliness’ or ‘intrinsic earliness’. Earliness per se is the difference in flowering times of varieties whose requirements of vernalization and photoperiod have been fulfilled. Photoperiod and vernalization response genes control flowering time of wheat in response to specific day length and temperature, whereas, earliness per se genes affect flowering time independent of environmental stimuli. Major vernalization and photoperiod genes may be regarded as ‘modifiers of earliness’ because they influence flowering only in response to certain environmental conditions, but earliness is determined by a minimum vegetative growth that can initiate floral primordia independent of external stimuli.

 Printed in Kazakhstan

The genes responsible for variation for ear emergence have been categorized as earliness per se (eps) [5]. The *Eps* genes are thought to be involved in different developmental phases including the transition from vegetative to reproductive growth, early and late spike development, stem elongation and heading, which determine grain yield components [5, 7]. They are also considered to be involved in the fine tuning of wheat flowering time within mega-environments [6] and are responsible for wide adaptation of wheat to different environments and regulation of flowering independent of both vernalization and photoperiod. Flowering time QTLs genes are found on almost all the wheat chromosomes [7] and eps generally causes differences of a few days (1–5) in flowering time [6]. However, knowledge of their identities, mechanism and the physiological and agronomic implications of different alleles/allelic combinations are poorly understood.

Over the years, wheat breeding has reduced genetic diversity with the replacement of traditional cultivars by modern high yielding varieties (FAO Document Repository). Genetic variation is prerequisite for crop improvement. Effective breeding programs for many agronomical traits require genetic variation, which has to be separable from non-genetic effects. It is widely believed that the genetic diversity of major crops, have suffered an overall reduction over time, primarily as a consequence of breeding processes and more recently due to the recurrent use of adapted germplasm and the adoption of breeding schemes that do not favour wide spread genetic recombination [8, 9].

Induced mutagenesis is a powerful tool to generate genetic variation from which desired mutants can be selected [11]. Over the past 80 years, it has been successfully applied to produce new mutant varieties in both seed and vegetatively-propagated crops [10,11, 12,13]. According to the FAO/IAEA Mutant Variety Database there are 3220 worldwide mutant plant varieties of 214 plant species as of 2014 (http://mvgs.iaea.org/). The use of mutagenesis in breeding involves the selection of individual mutants with improved traits and their incorporation into breeding programmes [10]. The release of mutant varieties has an enormous economic impact on agriculture and food production that is currently valued at billions of dollars from millions of cultivated hectares. Exploiting natural or induced genetic variability is a proven method for improving crops, and the use of mutagenesis to create novel variation is particularly valuable in major food crops that have restricted genetic variability [10]. Mutant lines developed in wheat and other crops have great potential for direct release or in cross-breeding programmes that develop new varieties [11,12]. Nuclear techniques contribute significantly in developing superior crop varieties in seed and vegetatively-propagated crops [12, 13].

The objective of this study was to develop new M_5 advanced wheat mutant lines and evaluate them along with parent for mapping density of dominant *VRN-A1* gene and *Esp-A1* candidate gene, and to determine effect of allele of *Eps-A1* candidate gene on flowering time in mutant germplasm and parent.

Materials and methods

Plant material and application of induced mutation

Seeds of uniform size of the spring bread wheat variety Zhenis (*Triticum aestivum L.)* were irradiated with 100 and 200 Gy radiation doses from a [^{60}Co] γ-ray radiation source at the Kazakh Nuclear Centre. Seeds were planted immediately after irradiation in order to raise M_1 plants. The M_1 generation was grown in the experimental field of the Kazakh Institute of Agricultural and Farming in Almaty. Single spikes were harvested from each plant to raise the M_2 generation, and the selection of the best lines from M_1 to M_5 was carried out based on individual plants. The M_3 and M_4 generation were grown with a randomized block design with three repetitions. The best lines were tested with their parent to select the best mutants. Seeds of best mutants were selected individually in each generation. It should be noted that standard wheat agriculture practices were followed. After harvesting M_5 plants, 15 advanced wheat M_5 lines in each type of mutant germplasm, 100 Gy radiation dose lines: 5(4), 6(4), 6(5), 6(13), 13(3), 18(5), (1), 24(2), 25(2), 26(6), 26(7), 26(9), 26(10), 30(1), 36(1), and 200 Gy radiation dose lines: 43(1), 43(3), 43(4), 45(1), 45(2), 45(3), 48(3), 49(2), 49(4), 49(6), 50(7), 51(1), 51(2), 51(8) and 53(2) were selected and used for evaluation of distribution frequency of *VRN-A1-F and Eps-A1* candidate genes.

Sampling and measurements

To measure productivity component, as the thousand grain weight, from the total number of grains (threshed 10 spikes), the weight in grams were measured in 2 samples, each 100 seeds, and thousand grain weight were calculated accordingly per line.

DNA extraction and SSR primer sources

Genomic DNA was isolated from leaves of 10-day-old seedlings of following CTAB extraction methods with our modifications [14]. DNA concentration was determined with the Eppendorf BioPhotometer plus.

Allelic variants at the *VRN* locus were detected using two gene-specific primers in a multiplex PCR assay, in which the primer pair:*VRN-A1-F*(5'TGAAAGGAAAAATTCTGCTCGT-3') *VRN-A1-R*(5'GGCAAACGGAATTACCAAATTG-GTAGATTCCGTTTGCC-3') are produced by a 798 bp fragment in the genotype with *VRN* alleles.

PCR was performed in a C1000 thermal cycler with a dual 48/48 fast-reaction module (Bio-Rad corporation, USA) and with 25 µl volume of 10xPCR buffer with 25 mM of $MgCl_2$, 200 µM of the dNTPs, 5 pmol of the primer *VRN-A1-F*, 2.5 pmol *VRN-1R* and *VRN-1 F*, one unit of Taq DNA polymerase, and 50–70 ng of template DNA. Thermocycling conditions were as follows: one cycle of 60 s at 95°C, 39 cycles of 20 s at 94°C, 30 s at 58°C, 30 s at 72°C, and then a final extension step at 72°C for 5 min. Amplified PCR products were separated on 1% agarose gel at 100 V stained with ethidium bromide and then visualized with UV light (Bio-Imaging System, Mini Bis Pro).

Allelic variants at the candidate gene *Eps-A^m1* locus were detected using two candidate gene-specific primer pairs in a multiplex PCR assay, in which the primer pair *Eps-A^m1-F*–(5'–CACATATCTG-GCACCCACA–3') and *Eps-A^m1-R*-(3'-TGCTCAT-GAGTTTTCC-TCTGAA-3') are produced by a 798 bp fragment in the genotype with *Eps-A^m1*-allele [7]. PCR reaction mixture (total 10 µl) consisted of 10xPCR buffer with 25 mM of $MgCl_2$, 10 mM of the dNTPs, 5 pmol of the primer *Eps-A^m1-F* and 2.5 pmol *Eps-A^m1-R*, 2U/µl of Taq DNA polymerase, and 100 ng of template DNA. Thermocycling conditions were as follows: one cycle of 30 s at 94°C, 30 s at 57°C, 30 s at 72°C, total 37 cycles. Amplification was carried out in the Multigene™ OptiMax Thermal Cycler with a dual 48/48 fast-reaction module (Labnet International, Inc). Amplified PCR products were separated on 1% agarose gel at 100 V stained with ethidium bromide and then visualized with UV light (the ENDURO™ GDS Gel Documentation System, Labnet International, Inc).

Recording of flowering time observations

Pot experiments were conducted in a greenhouse at Institute for Cereal Crops Improvement, Department of Molecular Biology and Ecology of Plants, Tel Aviv University to record flowering time observations. The experiment was set up in a factorial design with parent cv and mutant lines and 3 replications. Seeds were germinated in germination paper and seedlings were planted 4 days later. Thirty seeds were planted in each pot. Plants were grown in 2 cm deep of plastic pots (top diameter was 16 cm, a bottom diameter is of 12 cm, and a height of 18 cm) containing 2 kg of soil at constant temperature (23°C) and a long-day photoperiod (16 h) in a naturally lit greenhouse. The plants were watered once a week at 70% field capacity. Flowering time was estimated as number of days from the day of sowing to the day of spike emergence. To record the flowering date, wheat plots were inspected once in two days. It counts as the number of start flowering spikes. At this time, the number of leaves formed on the tiller was also recorded.

Statistical Analysis

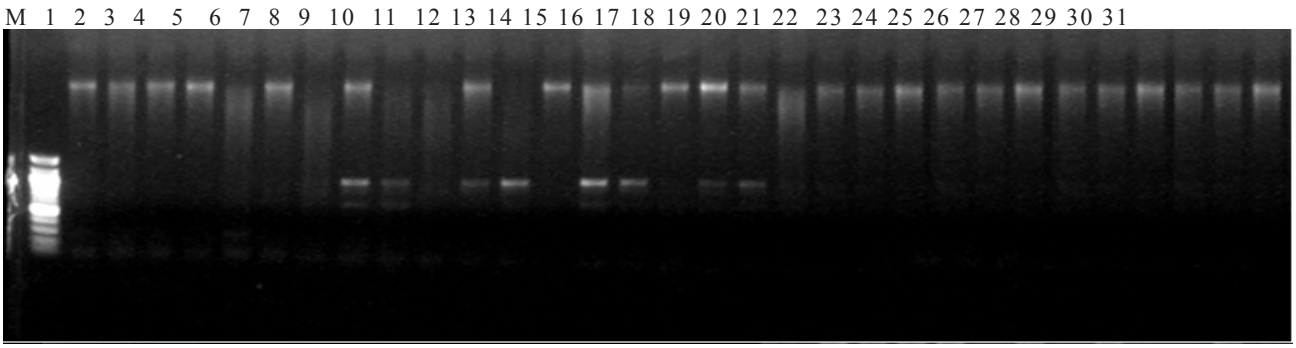
All data were evaluated in R 3.0.2 (R Core Development Team 2013). The simultaneous tests of general linear hypotheses, Dunnett Contrasts, were used for multiple comparisons of the means. Summary data are reported as mean values ± standard deviations. A p-value of less than 0.05 was considered statistically significant.

Results and discussion

One of the most important components of adaptation is flowering time, which is essentially determined by gene groups that regulate the vernalization requirement, i.e. the cold period to induce the transition from the vegetative to the generative phase (*VRN* genes) and the photoperiod sensitivity (*PPD* genes) and poorly defined 'earliness per se' (eps) or 'narrow sense' earliness genes [15].

In our study, SSR markers were used to detect the frequency of the dominant *VRN-A1* allele in M_5 mutant lines developed by using 100 and 200 Gy doses on genetic background of spring wheat cv. Zhenis along with parent. We revealed that allelic variation was observed at the dominant *VRN-A1* loci in the six 100 Gy lines, 24(2), 25(2), 26(7), 26(9), 30(1), 36(1), (40.0%) and two 200 Gy lines, 43(4), 43(3), (13.3%), all in all, eight M_5 mutant are different from the parent and other lines showing the presence of the dominant *VRN-A1* allele (Figure 1). Based on the frequency of the dominant *VRN-A1* allele in mutant lines, it is possible identify them as spring wheat (dominant). Thus, spring type allele of *VRN-A1* was present in 26.6% mutant lines, mostly in 100 Gy mutant lines. This finding may indicate the possible potential of induced mutagenesis for changing the development of the spring growth habit.

We observed that the two lines, 24(2) and 43(4) carrying dominant allele of *VRN-A1* had significantly higher thousand kernel weight (51.69±1.58 and 74.65±1.62 g, $p < 0.005$, respectively) than parent (38.08±3.67 g).



M, 1-cv.Zhenis, 1-5(4), 2-6(4),3-6(5), 4-6(13),5-13(3), 6-18(5), 7-(24(1), 8-24(2), 9-25(2), 10-26(6), 11-26(7), 12-26(9), 13-26(10), 14-30(1), 15- 36(1), 16-43(1), 17-43(3), 18-43(4), 19-45(1), 20-45(2), 21-45(3), 22-48(3), 23-49(2), 25-49(4), 26-49(6), 27-50(7), 28-51(1), 29-51(2), 30-51(8), 31-53(2).

Figure 1 – Amplification products obtained from M_5 mutant lines generated by treating 100 and 200 Gy radiation doses on cv. Zhenis of spring wheat genetic background and the parent in PCR, with the primer pair of the dominant *VRN-A1* gene.

It was reported, in wheat, the *VRN-A1*, *VRN-B1*, and *VRN-D1* genes have the greatest effect on the vernalization response, which are located on the long arm of the homologous chromosomes 5A, 5B, and 5D. Depending on the ratio of dominant and recessive alleles in the *VRN* genes in the three genomes of hexaploid wheat, cultivars could be distinguished as winter (recessive) or spring (dominant) varieties, while genotypes with the facultative habit have various combinations of dominant and recessive alleles. The dominant *VRN-A1a* allele has the most pronounced genetic effect on the development of the spring growth habit analysed the *VRN-A1* locus in 200 lines of hexaploid wheat with different growth habits (68 winter and 132 spring) and confirmed the presence of the recessive *VRN-A1* allele in winter wheat. In spring wheat, 55% carried the *VRN-A1a* allele and only 6% had the *VRN-A1b* allele. For the remaining lines, they confirmed the occurrence of the recessive *VRN-A1* allele characterized the *VRN-1* locus of 117 spring wheat cultivars from Argentina and California. The dominant *VRN-A1* allele was identified in 56.5% and *VRN-D1* in around 42% of cultivars, regardless of region of origin. Molecular characterization of vernalization response genes in 40 spring wheat cultivars from Western Canada showed that 34 carried the dominant *VRN-A1a* allele. Based on272 wheat cultivars from different geographical regions, it was concluded that difference in VRN genotypes are connected to their origin. In European wheat cultivars, the most frequent allele is *VRN-A1*, the dominant *VRN-B1* allele is of moderate frequency, and the dominant *VRN-D1* allele is very rare.

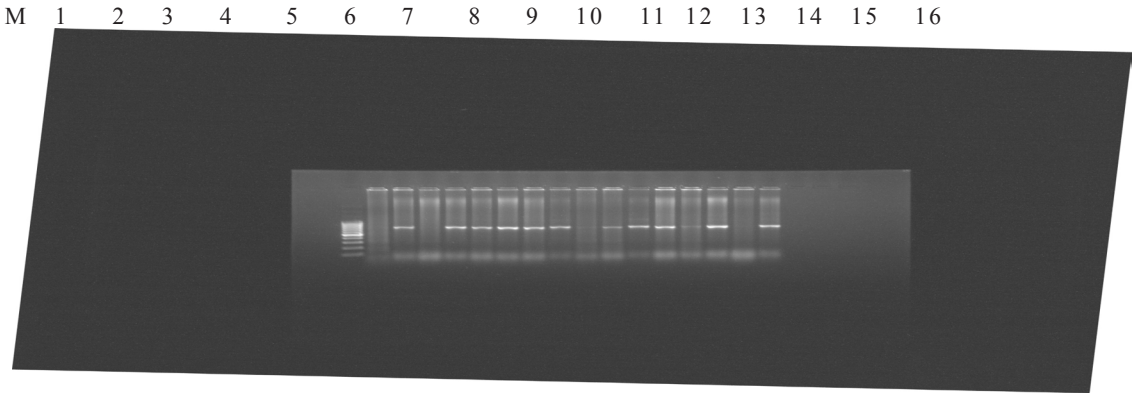
Genetic variation in emergence and maturation of wheat ears is the consequence of allelic variation at loci controlling the vegetative to floral transition, inflorescence development and stem extension. This variation has major implications for yield potential, abiotic and biotic stress tolerance/avoidance, interactions with agronomic interventions, and our ability for predictive breeding of germplasm adapted to specific environments [15].

We investigated genetic density for *Eps-A^{m}1* candidate gene in developed using 100 and 200 Gy radiation doses and genetic background of spring wheat cv. Zhenis by characterisation of allelic variation with PCR markers its flanking (Figure 2). If compare to the parent variety, we revealed that eleven 100 Gy M_5 mutant, 5(4), 6(5), 6(13), 13(3), 18(5), 24(1), 25(2), 26(6), 26(7), 26(10) and 36(1) (73.3%) had candidate gene *Eps-A^{m}1* allele (Figure 2A).

In the case of 200 Gy mutant germplasm, six M_5 lines, 45(1), 45(2), 49(2), 49(6), 50(7) and 51(1), (40.0%) showed amplification of this candidate gene the fragment (Figure 2B). This finding may indicate that in 200 Gy lines, the presence of candidate gene *Esp-A^{m}1* allele in numerical terms, as in the case of the VRN-A1 genes is less than those in 100 Gy radiation dose mutant germplasm.

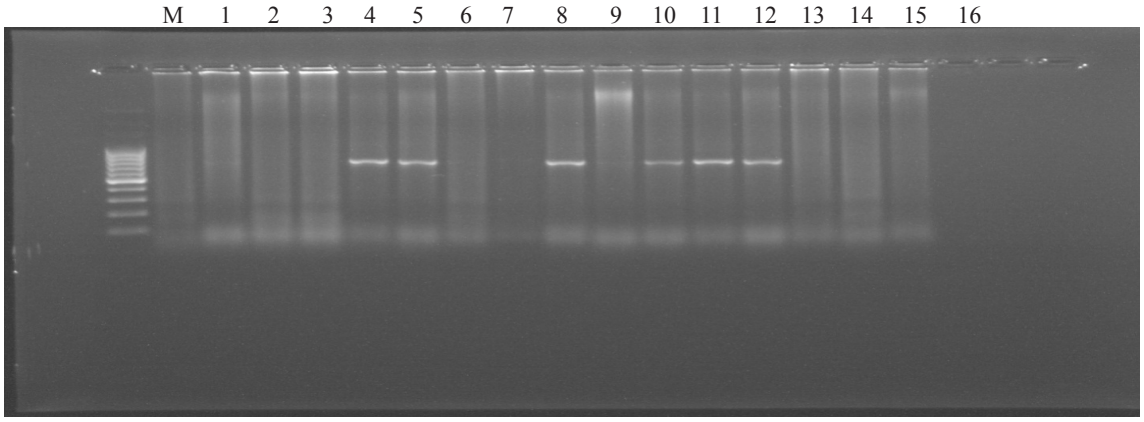
Comparison of the mutant lines with respect to density mapping in terms of allelic variants at the *VRN-A1* locus and candidate gene *Eps-A^{m}1* showed that among the M_5 lines identified as the genotypes are characterized by their difference in allele frequency from other lines and parent only one line 36(1) combined the two alleles, *VRN-A1* and candidate gene *Eps-A^{m}1*. This finding may suggest that induced mutagenesis may be effective tool to change allelic variation at the *Eps-A^{m}1* locus candidate gene.

A



M, 1-cv.Zhenis, 2-5(4),3-6(4), 4-6(5), 5-6(13), 6-13(3), 7-18(5), 8-24(1), 9-24(2), 10-25(2), 11-26(6), 12-26(7), 13-26(9), 14-26(10), 15-30(1), 16-36(1).

B



M, 1-cv.Zhenis, 2-43(1), 3-43(3), 4-43(4), 5-45(1), 6-45(2), 7-45(3), 8-48(3), 9-49(2), 10-49(4), 11-49(6), 12-50(7), 13-51(1), 14-51(2), 15-51(8), 16-53(2).

Figure 2 – Amplification products obtained from M_5 mutant lines of spring wheat developed with 100 Gy (A) and 200 Gy (B) and cv. Zhenis in PCR, with the primer pair of the candidate gene *Eps-A^{m}1*.

Genetic variation in emergence and maturation of wheat ears is the consequence of allelic variation at loci controlling the vegetative to floral transition, inflorescence development and stem extension. This variation has major implications for yield potential, abiotic and biotic stress tolerance/avoidance, interactions with agronomic interventions, and our ability for predictive breeding of germplasm adapted to specific environments. The timing of ear emergence is fundamental to plant survival in that it allows plant species to flower at the most suitable period which will allow pollination, seed set and dispersal [15].

Effect of the candidate gene *Eps-A^{m}1* allele was determined on the flowering time of wheat developmental phases. Mutant populations differed in their flowering times, having less means, particularly 100 Gy lines (87±12.86 days) than parent (115±18.23) (Table 1). Such observed differences among genotypes could be attributed to effect of high mapping density of candidate gene *Eps-A^{m}1* alleles.

This finding may indicate the potential of induced mutagenesis for changing the flowering time, independently of vernalization and photoperiod, and are important for the fine tuning of flowering time and for the wide adaptation of wheat to different environments. Generated M_5 mutant lines of spring wheat on cv. Zhenis parent grown in Kazakhstan as genetic background can be classified as new sources having genetic potential to synchronize their flowering time with favourable environmental conditions.

Varietal differences in flowering time other than those due to photoperiod and vernalization response have been observed in wheat [15]. Such genetic differences are termed ‘‘earliness per se’’ , ‘narrow-sense earliness’ or ‘intrinsic earliness’.

Table 1 – Descriptive statistics of flowering time among spring wheat mutant lines and cv. Zhenis parent

Variable	cv. Zhenis (parent)	100 Gy M_5 lines	200 Gy M_5 lines
Lines numbers	30	30	30
Flowering days (from sowing to flowering days)	115±18.23	87±12.86	103±15.45
Number of main spike at the beginning of flowering	94±52	120±52	97±36

Earliness per se is the difference in flowering times of varieties whose requirements of vernalization and photoperiod have been fulfilled. Photoperiod and vernalization response genes control flowering time of wheat in response to specific day length and temperature, whereas, earliness per se genes affect flowering time independent of environmental stimuli. Major vernalization and photoperiod genes may be regarded as 'modifiers of earliness' because they influence flowering only in response to certain environmental conditions, but earliness is determined by a minimum vegetative growth that can initiate floral primordia independent of external stimuli.

Earliness per se is highly heritable and can, therefore, be effectively utilized in breeding programs to shorten wheat's life cycle independent of other environmental factors known to modify flowering time.

This study provides effective approach of induced mutagenesis with gamma rays treatment for generation of considerable genetic variability between and within mutant populations of spring common wheat variety Zhenis. It allows to identify of new M_5 mutant lines with allelic variation of the dominant *VRN-A1* and earliness related *Esp-A1* candidate genes in advanced wheat mutant lines and effect of allele on flowering time. Developed mutant wheat germplasm offers great possibilities for exploitation of physiological and biochemical aspects of the three genetic constituents of flowering and maturity time in wheat.

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