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Allelic variation at the *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, *Vrn-B3* and *Ppd-D1a* loci of Pakistani spring wheat cultivars

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Abstract Flowering time in bread wheat (*Triticum aestivum* L.) is controlled by vernalization and photoperiod response, and earliness *per se* genes. The genetic basis of flowering time has not been investigated in Pakistani bread wheat. This study was, therefore, conducted to determine the allelic composition at *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, *Vrn-B3* and *Ppd-D1a* loci of 59 Pakistani spring bread wheat cultivars. These cultivars, along with 4 isogenic lines for vernalization genes were characterized with previously reported DNA markers designed for detecting allelic variation at 4 *Vrn* (Vernalization) and 1 *Ppd* (Photoperiod) loci. Spring habit *Vrn-A1a* allele was found in 36% cultivars either alone or with spring habit *Vrn-B1* and *Vrn-D1* alleles. Two wheat cultivars had the dominant *Vrn-A1c* allele, whereas none of the cultivars had *Vrn-A1b*. Spring habit *Vrn-B1* was the most frequent allele (64%) present either alone or with *Vrn-A1a*, *Vrn-A1c* and *Vrn-D1*. Spring habit *Vrn-D1* was found in 61% cultivars. *Vrn-D1* was singly found in 25% cultivars and along with *Vrn-B1* in 29% cultivars. Dominant *Vrn-B3* was absent in all cultivars studied. All cultivars except Era had the photoperiod insensitive allele *Ppd-D1a*. We did not find any association between the flowering time and *Vrn* allelic composition of the studied cultivars. This indicated that the partial vernalization requirement of cultivars with *Vrn-B1* and *Vrn-D1* alleles is probably fulfilled during Pakistani growing season. Earliness *per se* and the photoperiod sensitive loci other than *Ppd-D1* need to be investigated to further understand the genetic basis of flowering time in Pakistani wheat.

Keywords: photoperiod, spring growth habit, *Triticum aestivum*, vernalization

INTRODUCTION

The field performance of a crop is mainly determined through its developmental pattern (Richards, 1996). The final grain yield of wheat is not only determined by genes that directly control yield and yield components but also by genes that confer tolerance/resistance to various abiotic and biotic stresses (Slafer, 2003). Global increase in wheat yield has been achieved by modifying its developmental pattern so as to best suit the growing conditions of a particular region. The adaptability of wheat to a particular set of environmental conditions requires adjustments in life cycle such that flowering and maturity occur at the most appropriate times (Cockram et al. 2007). A thorough understanding of the genetic factors governing adaptability aids in developing crop varieties specifically adapted to different environments, thereby ensuring maximum crop production (Ortiz-Ferrara et al. 1995).

Three genetic systems, including vernalization (*Vrn*) response, photoperiod (*Ppd*) sensitivity, and earliness *per se*, control the growth and developmental phases of wheat (Herndl et al. 2008). Wheat yield potential in different environments is, therefore, mainly determined by these three systems and their interactions with the temperature of that environment (Gororo et al. 2001). Vernalization accelerates flowering/maturity in sensitive wheat when exposed to cold temperatures (Cockram et al. 2007). Four major loci *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Vrn-D5* control vernalization response in wheat (Iwaki et al. 2000). The presence of recessive alleles at all these loci confers winter growth habit, whereas presence of one or more dominant alleles at these loci results in spring growth habit. The dominant *Vrn* alleles have differential sensitivity to vernalization. Wheat cultivars with dominant allele of

Vrn-A1 are insensitive to vernalization, whereas those having dominant alleles at *Vrn-B1*, *Vrn-D1* or *Vrn-D5* exhibit low sensitivity to vernalization (Shindo and Sasakuma, 2002). *Vrn-A1* masks the low vernalization sensitivity of the other dominant alleles.

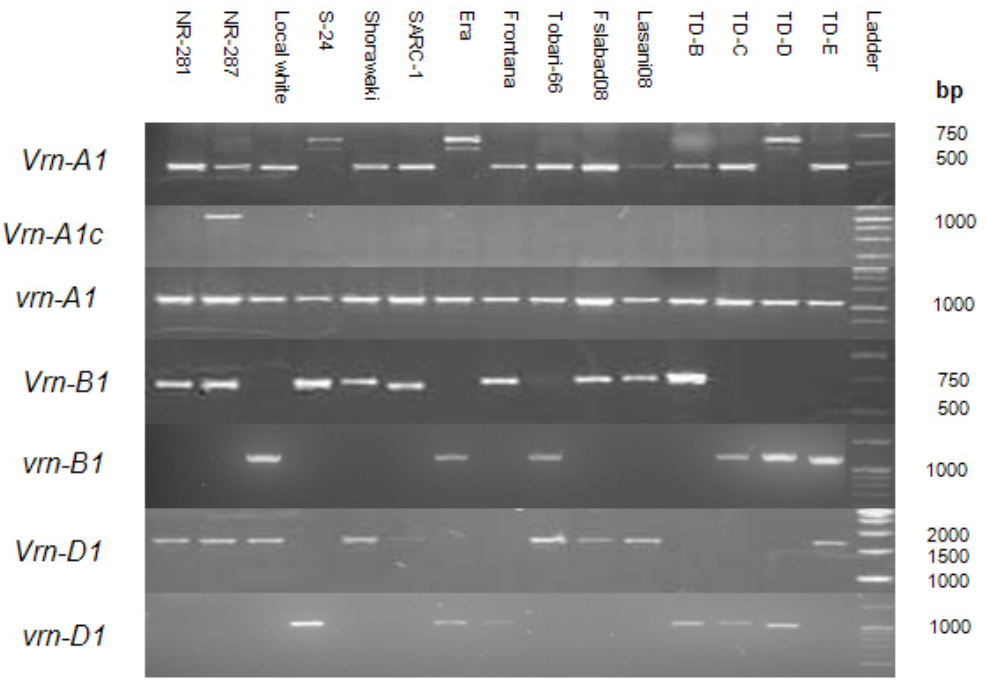

Fig. 1 PCR amplified DNA fragments using primers for major vernalization genes in selected Pakistani wheat.

Vernalization response variation of the dominant *Vrn* alleles causes differences in the flowering time of spring wheat. Therefore, wheat cultivars with dominant *Vrn-A1* allele flower the earliest, whereas those with dominant *Vrn-D1*, *Vrn-D5* and/or *Vrn-B1* flower later, respectively, under non-vernalizing conditions (Goncharov, 2004). Besides altering flowering time, different combinations of dominant *Vrn* alleles may also cause variation in plant height and yield components in wheat (Stelmakh, 1992; Stelmakh, 1998). The presence of two dominant alleles at major vernalization loci confers early maturity as well as higher yield potential. A combination of dominant *Vrn-A1a*, *Vrn-B1* and *Vrn-D1* alleles results in earliest maturity but low yielding wheat cultivars. These findings indicate that early maturing spring wheat cultivars with acceptable grain yield potential may be developed if specific dominant *Vrn* alleles are combined in a genotype. Partial vernalization requirement may also be beneficial for improving frost tolerance in wheat (Iwaki et al. 2001).

Photoperiod response determines flowering time, and hence adaptation of wheat to different agro-climatic conditions. A photoperiod insensitive wheat cultivar immediately switches to reproductive growth with a rise in temperature in the spring, whereas a photoperiod sensitive cultivar continues its vegetative phase until the day length sufficiently increases to satisfy its photoperiod requirement (Worland and Snape, 2001). A lack of fulfilment of photoperiod requirement in sensitive cultivars results in delayed flowering, the magnitude being determined by the presence of specific photoperiod response genes and the latitude of the growing region. Photoperiod response in wheat is genetically controlled by allelic variation at the *Ppd-A1*, *Ppd-B1* and *Ppd-D1* loci that are located on homologous group 2 chromosomes (Snape et al. 2001). Similar to *Vrn* gene system, dominant alleles of *Ppd* genes confer day length insensitivity, whereas the presence of recessive alleles results in day length sensitivity (Dyck et al. 2004).

Table 1. Primer sequences, annealing temperatures and expected PCR product sizes for detecting alleles at major *Vrn* and *Ppd-D1* loci in wheat.

Locus	Allele	Primers	Primer sequence (5′—3′)	Expected PCR product size (bp)	Annealing Temperature (°C)
	Vrn-A1a Vrn-A1b vrn-A1	VRN1AF VRN1R	GAAAGGAAAAATTCTGCTCG TGCACCTTCCC(C/G)CGCCCCAT	650+750 ≈480 ≈500	55
Vrn-A1	*Vrn-A1c*	Intr1/A/F2 Intr1/A/R3	AGCCTCCACGGTTTGAAAGTAA AAGTAAGACAACACGAATGTGAGA	1170	57
	vrn-A1	Intr1/C/F Intr1/AB/R	GCACTCCTAACCCACTAACC TCATCCATCATCAAGGCAAA	1068	57
Vrn-B1	*Vrn-B1*	Intr1/B/F Intr1/B/R3	CAAGTGGAACGGTTAGGACA CTCATGCCAAAAATTGAAGATGA	709	58
	vrn-B1	Intr1/B/F Intr1/B/R4	CAAGTGGAACGGTTAGGACA CAAATGAAAAGGAATGAGAGCA	1149	58
Vrn-D1	*Vrn-D1*	Intr1/D/F Intr1/D/R3	GTTGTCTGCCTCATCAAATCC GGTCACTGGTGGTCTGTGC	1671	61
	vrn-D1	Intr1/D/F Intr1/D/R4	GTTGTCTGCCTCATCAAATCC AAATGAAAAGGAACGGAGCG	997	56
Vrn-B3	*Vrn-B3*	VRN4-B-INS-F VRN4-B-INS-R	CATAATGCCAAGCCGGTGAGTAC ATGTCTGCCAATTAGCTAGC	≈1200	63
	vrn-B3	VRN4-B-NOINS-F VRN4-B-NOINS-R	ATGCTTTCGCTTGCCATCC CTATCCCTACCGGCCATTAG	≈1140	57
	Ppd-D1a	Ppd-D1_F	ACGCCTCCCACTACACTG	414	
Ppd-D1		Ppd-D1_R1	TTGGTTCAAACAGAGAGC		54
	ppd-D1a	Ppd-D1_R2	CACTGGTGGTAGCTGAGATT	288	

The spring growth habit at *Vrn-A1* locus of bread wheat is known to result from mutations in the promoter region (Yan et al. 2004), whereas spring growth habit at *Vrn-B1* and *Vrn-D1* loci is determined by large deletions within the first intron (Fu et al. 2005). These findings have led to the development of allele specific DNA markers to detect the presence/absence of *Vrn* alleles in wheat germplasm, and to understand their role in the adaptation of wheat in different geographical regions (Yan et al. 2004; Fu et al. 2005). Beales et al. (2007) demonstrated that photoperiod insensitivity at the *Ppd-D1a* locus of wheat was due to a 2089-bp deletion upstream of the coding region. They subsequently designed gene-specific primers for detecting allelic variation at this locus.

Presently, there is no knowledge of whether vernalization response genes affect flowering/maturity times in Pakistani wheat cultivars under field conditions. Moreover, the *Vrn* genes of Pakistani wheat cultivars (except Inqalab-91) are not known. Detection of allelic variation at the major *Vrn* and *Ppd* loci will improve our understanding of the genetic basis of flowering and maturity times of Pakistani wheat cultivars. This may also assist wheat breeders in the incorporation of desirable *Vrn* genes combinations into Pakistani wheat as a means to escape biotic and abiotic stresses.

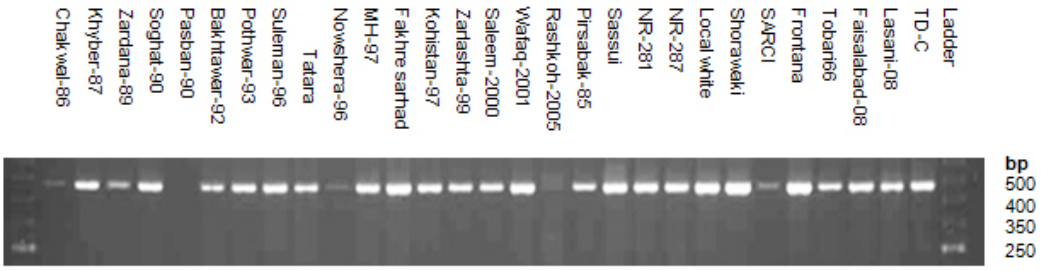

Fig. 2 Electrophoresis of PCR products differentiating *Vrn-A1b* and *vrn-A1* alleles (20 bp difference) in Pakistani wheats on 2.5% high resolution agarose gel.

MATERIALS AND METHODS

Fifty nine Pakistani wheat cultivars, released from 1970 to 2008, and 4 isogenic lines of *Vrn* genes namely, Triple Dirk-B (*vrn-A1 Vrn-B1 vrn-D1*), Triple Dirk-D (*Vrn-A1 vrn-B1 vrn-D1*), Triple Dirk-E (*vrn-A1 vrn-B1 Vrn*-D1) and Triple Dirk-C (*vrn-A1 vrn-B1 vrn*-D1) were selected for this study. Seeds of the Pakistani wheat cultivars were provided by National Coordinated Wheat Program, National Agricultural Research Center (NARC), Islamabad, Pakistan. Genomic DNA was extracted from 3-5 mature dry seeds following, with slight modification, the procedure developed by Kang et al. (1998). DNA samples were quantified and diluted to working concentration of approximately 50ng/µl. The 20 µl PCR reaction mixture contained 1 x PCR buffer with $(NH_4)_2SO_4$, 3mM $MgCl_2$, 0.2mM dNTPs mix, 10 pmol each of the reverse and forward primers, one unit of *Taq* DNA Polymerase (Fermentas, Life Sciences) and DNA template of 50 ng. Polymerase chain reaction (PCR) primers designed by Yan et al. (2004), Fu et al. (2005), Yan et al. (2006) and Beales et al. (2007), given in Table 1, were used for detecting allelic variation at *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, *Vrn-B3* and *Ppd-D1a* loci. PCR was programmed to initially denature DNA at 94ºC for 5 min, followed by 35 cycles at 94ºC for 30 sec, 54-63ºC for 30 sec, and 72ºC for 2 min. Final extension was at 72ºC for 10 min. PCR products were separated on 1.5% agarose gel stained with ethidium bromide and subsequently visualized. The difference between *Vrn-A1* and *Vrn-A1b* was resolved by electrophoresing the PCR products on 3% high resolution agarose. PCR reactions for all primer pairs were repeated to confirm the *Vrn* and *Ppd-D1a* genotype of all varieties. Heading data were recorded on single row plots (non-replicated) of all varieties for two years.

RESULTS AND DISCUSSION

VRN1AF and VRN1R primers (*Vrn-A1*) amplified two PCR products similar in sizes to that of TD-D in 21 of the 59 wheat cultivars/lines (Figure 1), suggesting that these have the spring habit *VrnA1a* allele at *Vrn-A1* locus. A PCR product similar in size to that of TD-C was observed for the remaining 38 cultivars/lines (Figure 2). This indicated that these carry the winter habit allele *Vrn-A1*. PCR products of cultivars/lines not having *Vrn-A1a* allele did not show size variation on 3% high resolution agarose gel

Table 2. Allelic variation at the major loci governing vernalization response in Pakistani wheats.

Allele combination	Wheat cultivars/lines
Vrn-A1a Vrn-B1 Vrn-D1 *vrn-B3*	Shahkar-95
Vrn-A1c Vrn-B1 Vrn-D1 *vrn-B3*	Pavon-76, NR-287
Vrn-A1a Vrn-B1 *vrn-D1 vrn-B3*	Punjab-76 , Tandojam-83 , Sarsabz, Shalimar-88 , Inqilab-91, Sariab-92, Kiran-95, Punjab-96, Auqab-2000, Moomal-2002, SH-2003, Sehr-2006, Shafaq-2006, S-24
Vrn-A1a *vrn-B1* ***Vrn-D1*** *vrn-B3*	Blue silver
Vrn-A1a *vrn-B1 vrn-D1 vrn-B3*	Chakwal-97, Marvi-2000, GA-2002, Era, SA-42
vrn-A1 ***Vrn-B1 Vrn-D1*** *vrn-B3*	Lyallpur-73, Pak-81, Kohinoor-83, Zardana-89, Soghat-90, Pothwar-93, Suleman-96, Nowshera-96, MH-97, Fakhre Sarhad , Kohistan-97, NARC-2009 , NR-281, Shorawaki, SAARC-I , Faisalabad-2008 , Lasani-2008
vrn-A1 ***Vrn-B1*** *vrn-D1 vrn-B3*	Barani-70, Zarghoon-79, Rashkoh-2005, Frontana
vrn-A1 vrn-B1 ***Vrn-D1*** *vrn-B3*	Yecora-70, Lu-26, Pirsabak-85, Khyber-87, Chakwal-86, Pasban-90, Bakhtawar-92, Tatara, Zarlashta-99, Saleem-2000, Wafaq-2001, Pirsabak-05, Sassui, Local white, Tobari-66

(Figure 2). This indicated that spring habit *Vrn-A1b* allele was absent in the cultivar/line studied. The primers Intr/A/F2 and Intr/A/R3 amplified PCR products of 1170 bp in Pavon-76 and NR-287, indicating the presence of *Vrn-A1c* allele in these genotypes.

Intr1/B/F and Intr1/B/R3 (*Vrn-B1* primers) amplified a PCR product similar in size to that of TD-B in 38 of the cultivars/lines (Figure 1), indicating the presence of spring habit allele at the *Vrn-B1* locus. No amplification was observed in the remaining genotypes, suggesting the presence of winter habit allele at *Vrn-B1*. Intr1/B/F and Intr1/B/R4 (*vrn-B1* primers) amplified PCR fragments in 21 cultivars/lines, confirming that these carry winter habit allele at *vrn-B1* locus. Intr1/D/F and Intr1/D/R3 (*Vrn-D1* primers) produced similar size PCR fragment to that of TD-E (1650 bp) in 35 of the cultivars/lines, indicating that these carry spring habit allele at *Vrn-D1* locus. No amplification was observed in the remaining 24 cultivars/lines, suggesting the presence of winter habit allele at *Vrn-D1*. The presence of winter habit allele V*rn-D1* was also confirmed using primers Intr1/D/F and Intr1/D/R4, which produced a PCR fragment of approx. 997 bp in the 24 cultivars/lines.

Primers VRN4-B-INS-F and VRN4-B-INS-R failed to amplify a PCR product in all cultivars/lines studied, indicating the absence of spring habit allele at *Vrn-B3* locus. This was further confirmed with primers VRN4-B-NOINS-F and VRN4-B-NOINS-R that amplified an approximately 1140 bp fragment in all cultivars/lines. PCR using primers Ppd-D1_F, Ppd-D1_R1 and Ppd-D1_R2 in a single reaction produced a fragment of 414 bp only in 'Era', indicating the presence of photoperiod sensitive allele at *Ppd-D1* locus. A PCR product of 288 bp was amplified in the remaining cultivars/lines, suggesting that these carry the photoperiod insensitive allele at *Ppd-D1* locus. We did not find any association between different combinations of *Vrn* alleles and flowering time of the studied wheat cultivars/lines (Table 1, Table 2 and Table 3).

Results of the present study revealed that different combinations of *Vrn* alleles control the spring growth habit in Pakistani wheat. The spring habit allele *Vrn-A1a* was found in 36% of the Pakistani wheat cultivars studied. However, only 9% of the cultivars had *Vrn-A1a* as their sole spring growth habit allele. Spring wheat genotypes possessing *Vrn-A1a* allele do not have a vernalization requirement (Shindo and Sasakuma, 2002) and are, therefore, early flowering than those having the slight responsive *Vrn-B1* or *Vrn-D1* alleles in non-vernalizing conditions. The highest frequency (85%) of *Vrn-A1a* has been reported in Canadian spring wheat (Iqbal et al. 2007), followed by 69% in wheat germplasm from Pacific Northwest region of the USA (Santra et al. 2009), 50% in the spring wheat cultivars of the USA and Argentina (Yan et al. 2004) and 44% in Chinese wheat (Zhang et al. 2008). The dominant allele *Vrn-A1c* was found in the advanced breeding line NR-287 and Pavon-76 only. No deletion in the intron 1 of *Vrn-A1* was found in these two genotypes, indicating the duplication of this región.

Table 3. Days to heading of 59 Pakistani wheat cultivars during 2008-09 and 2009-10.

	Days to heading			Days to heading	
Cultivar	**2008-09**	**2009-10**	**Cultivar**	**2008-09**	**2009-10**
Barani-70	110	115	Koshistan-97	115	120
Lyallpur-73	110	116	Zarlashta-99	115	119
Yecora-70	101	110	Auqab-2000	106	116
Punjab-76	113	116	Saleem-2000	108	116
Lu-26	101	111	Marvi-2000	105	115
Pavon-76	114	NA	Wafaq-2001	112	114
Zarghoon-79	127	120	GA-2002	104	114
Pak-81	123	116	Moomal-2002	104	117
Kohinoor-83	109	116	SH-2003	104	113
Tandojam-83	109	NA	Raskoh-2005	103	115
Pirsabak-85	109	NA	Pisrsabak-05	107	115
Sarsabz	112	115	Sehar-2006	102	107
Chakwal-86	114	110	Shafaq-2006	94	103
Khyber-87	109	117	Sassui	113	115
Shalimar-88	102	112	NARC-09	113	116
Zardana-89	110	114	NR-281	105	NA
Soghat-90	110	115	NR-287	106	NA
Pasban-90	115	116	Local White	116	112
Inqilab-91	106	113	S-24	106	NA
Sariab-92	110	117	Shorawaki	158	NA
Bakhtawar-92	109	116	SARC-1	112	NA
Pothowar-93	140	120	Era	148	NA
Kiran-95	105	NA	Frontana	116	NA
Shahkar-95	99	115	Tobari-66	111	NA
Suleman-96	105	111	Faisalabad-08	105	114
Tatara	105	115	Lasani-08	97	114
Nowshera-96	109	118	Punjab-96	106	117
MH-97	106	117	SA-42	93	109
Chakwal-97	105	116	Blue Silver	94	114
Fakhr-e-Sarhad	NA*	118			

*Not available

Fu et al. (2005) also reported both presence and absence of intron 1 deletion in IL 369, a hexaploid land race of wheat from Afghanistan. Among hexaploid wheats, *Vrn-A1c* has been previously found only in two land races from Afghanistan and Egypt (Yan et al. 2004). *Vrn-A1b* allele was not found in any of the Pakistani spring wheat tested in the present study. *Vrn-B1* was the most frequent (64%) among the spring habit alleles in Pakistani wheats. It was present as a sole spring habit allele in 4 cultivars. However, the frequency of its occurrence was relatively higher with either *Vrn-A1a* or *Vrn-D1* (24% and 29%, respectively). *Vrn-D1* was the most frequent (25%) singly present spring habit allele in Pakistani wheat cultivars/lines. It was also present along with spring habit *Vrn-B1* allele in 29% of Pakistani wheat tested. The high frequency of *Vrn-D1* in Pakistani wheat is probably due to the greater use of CIMMYT material both as direct selection and as parental lines in developing new wheat cultivars. Wheat material developed at CIMMYT has a higher frequency of spring habit *Vrn-D1* allele (Van Beem et al. 2005). The spring habit allele *Vrn-B3* was not found in Pakistani wheat cultivars/lines. Zhang et al. (2008) reported a low frequency (2 of 278) of spring habit allele *Vrn-B3* in Chinese wheats. All Pakistani wheat cultivars/lines tested had the photoperiod insensitive *Ppd-D1a* allele.

We observed a high frequency of spring growth habit *Vrn* alleles, *Vrn-B1* and *Vrn-D1* in Pakistani wheat cultivars. These alleles confer slight vernalization sensitivity in wheat genotypes and result in delayed flowering in non-vernalizing growing conditions. However, in the present study we did not find any association between different combinations of *Vrn* alleles and flowering time of Pakistani wheat cultivars/lines. This indicated that the partial vernalization requirement is probably fulfilled under Pakistani wheat growing conditions. Spring wheat is grown in fall in Pakistan which follows a mild winter. The low temperatures during winter probably fulfill the vernalization requirement of wheat varieties with *Vrn-B1* and *Vrn-D1* alleles, resulting in early flowering than would be expected under non-vernalizing conditions. Our results have provided a basis for understanding the genetic basis of flowering time in Pakistani wheats. Further studies are needed to investigate the role of other genetic systems, especially earliness *per se* in controlling flowering time and adaptation in Pakistani wheat. Pakistan has diverse agro-climatic zones, some experiencing terminal drought and heat stresses. Understanding the genetic basis of flowering time and hence maturity time will facilitate in developing new wheat cultivars with altered flowering time that can avoid biotic and abiotic stresses during late grain filling period.

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