RESEARCH ARTICLE

Genetics of flowering time in bread wheat *Triticum aestivum*: complementary interaction between vernalization-insensitive and photoperiod-insensitive mutations imparts very early flowering habit to spring wheat

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Abstract

Time to flowering in the winter growth habit bread wheat is dependent on vernalization (exposure to cold conditions) and exposure to long days (photoperiod). Dominant *Vrn-1* (*Vrn-A1*, *Vrn-B1* and *Vrn-D1*) alleles are associated with vernalization-independent spring growth habit. The semidominant *Ppd-D1a* mutation confers photoperiod-insensitivity or rapid flowering in wheat under short day and long day conditions. The objective of this study was to reveal the nature of interaction between *Vrn-1* and *Ppd-D1a* mutations (active alleles of the respective genes *vrn-1* and *Ppd-D1b*). Twelve Indian spring wheat cultivars and the spring wheat landrace Chinese Spring were characterized for their flowering times by seeding them every month for five years under natural field conditions in New Delhi. Near isogenic *Vrn-1 Ppd-D1* and *Vrn-1 Ppd-D1a* lines constructed in two genetic backgrounds were also phenotyped for flowering time by seeding in two different seasons. The wheat lines of *Vrn-A1a Vrn-B1 Vrn-D1 Ppd-D1a*, *Vrn-A1a Vrn-B1 Ppd-D1a* and *Vrn-A1a Vrn-D1 Ppd-D1a* (or *Vrn-1 Ppd-D1a*) genotypes flowered several weeks earlier than that of *Vrn-A1a Vrn-B1 Vrn-D1 Ppd-D1b*, *Vrn-A1b Ppd-D1b* and *Vrn-D1 Ppd-D1b* (or *Vrn-1 Ppd-D1b*) genotypes. The flowering time phenotypes of the isogenic vernalization-insensitive lines confirmed that *Ppd-D1a* hastened flowering by several weeks. It was concluded that complementary interaction between *Vrn-1* and *Ppd-D1a* active alleles imparted super/very-early flowering habit to spring wheats. The early and late flowering wheat varieties showed differences in flowering time between short day and long day conditions. The flowering time in *Vrn-1 Ppd-D1a* genotypes was hastened by higher temperatures under long day conditions. The ambient air temperature and photoperiod parameters for flowering in spring wheat were estimated at 25°C and 12 h, respectively.

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Introduction

Bread wheat, *Triticum aestivum*, is the leading food grain crop of the world, areawise and productionwise. Since its vegetative growth and seed productivity are sensitive to high temperatures, wheat is cultivated in areas of temperate and semitemperate agroclimates (Kumar *et al.* 2012). Two types of bread wheat varieties are in cultivation. The so called winter wheat plant type varieties are grown where young crop faces extended period of frosty weather conditions or undergoes vernalization (Kumar *et al.* 2012). Winter wheat crops develop flowering competence in response to vernalization (Flood and Halloran 1984; Goncharov 2004; Trevaskis *et al.* 2007). Spring wheat plant varieties are cultivated in the rest of the cold agroclimates (Cochram *et al.* 2007; Baga *et al.* 2009; Eagles *et al.* 2010). Spring wheat varieties have been bred to adapt enormous variation in crop season length experienced in diverse areas of cultivation. This has become possible because of the flowering time of spring wheats is much shorter than that of winter wheats (Cochram *et al.* 2007; Trevaskis 2010).

Vernalization-insensitivity is responsible for the early flowering characteristic of spring wheats (Pugsley 1971). Flowering in wheat requires the Vrn-1 function specified by

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the *vrn-A1*, *vrn-B1*, *vrn-D1* and *vrn-D4* genes borne on the group 5 chromosomes, in the A, B and D subgenomes of the allo-hexaploid bread wheat genome (Yan *et al.* 2004; Fu *et al.* 2005; Baga *et al.* 2009; Eagles *et al.* 2009; Oliver *et al.* 2009; Shimada *et al.* 2009; Trevaskis 2010; Yoshida *et al.* 2010; Rousset *et al.* 2011). The Vrn-1 protein is a MADS box transcription factor highly homologous to APETALA-1, CAULIFLOWER and FRUITFUL which regulate the transition of shoot meristem from vegetative-to-reproductive phase, in *Arabidopsis thaliana*. *Vrn-1* is required for the establishment and maintenance of the floral meristem identity at tiller apex in wheat plants (Danyluk *et al.* 2003; Trevaskis *et al.* 2003; Yan *et al.* 2003; Preston and Kellog 2008). In winter wheat crops, *vrn-1* genes remain repressed until induced in response to vernalization. In the spring wheats, Vrn-1 synthesis occurs constitutively because of mutational loss of the repressor binding site in the regulatory region of one or more *vrn-1* gene(s) (Loukoianov *et al.* 2005). This kind of dominant *cis*-mutation in any one of the *vrn-1* homeologue genes is sufficient for the acquirement of spring habit and general loss of dependence on vernalization for flowering (Preston and Kellog 2008; Greenup *et al.* 2009). The dominant mutant (or active) alleles *Vrn-A1a*, *Vrn-A1b* and *Vrn-A1c* are known in the *vrn-A1* gene, *Vrn-B1* in *vrn-B1* gene and *Vrn-D1* in *vrn-D1* gene (Yan *et al.* 2003, 2004; Fu *et al.* 2005). Combinations of these alleles are known to impart different degrees of earliness in flowering to the spring wheat.

The winter-wheat and spring-wheat varieties can be photoperiod-sensitive or photoperiod-insensitive. The photoperiod-insensitive varieties are early flowering in short day (SD) conditions as compared to their counterpart photoperiod-sensitive varieties. Winter wheat crops, following their vernalization, require exposure to long day (LD; >10 h light) for several to many weeks before their plants flower. Photoperiod-sensitivity is controlled by *Ppd-A1*, *Ppd-B1* and *Ppd-D1* genes of the group 2 chromosomes (Kato and Yokoyama 1992; Worland *et al.* 1998; Dubcovsky *et al.* 2006; Yang *et al.* 2009; Guo *et al.* 2010; Bentley *et al.* 2011). The *Ppd* genes specify a type of pseudo response regulator (PRR) protein which is involved in the activation of the photoperiod pathway leading to induction of the *vrn-3* gene (located on 7B chromosome of wheat) which is a homologue of the *FLOWERING LOCUS T* (*FT*) of *A. thaliana*. Expressed in leaves the TaFT or Vrn-3 is the flowering signal (florigen) that moves from leaves to apices and induces meristem identity genes involved in the initiation of flowering (Yan *et al.* 2006; Bonnin *et al.* 2008; Shimada *et al.* 2009). The wildtype *Ppd* genes become active only on sufficient exposure of plants to LD. Mutations in *Ppd-B1* and *Ppd-D1* have been implicated in the main in the photoperiod-insensitivity in wheats (Mohler *et al.* 2004; Beales *et al.* 2007). The semidominant *Ppd-D1a* mutation has been identified as the major source of earliness in wheat varieties worldwide. *Ppd-D1a* allele is misexpressed to synthesize PRR protein and thereby causes induction of the Vrn-3 florigen, under both SD and LD conditions (Beales *et al.* 2007). Vrn-3 upregulates *Vrn-1* expression such that it reaches above the threshold level required for the induction of flowering (Yan *et al.* 2006; Li and Dubcovsky 2008; Winfield *et al.* 2009). Like the dominant *Vrn-1* mutations, a dominant retrotransposon insertion allele is known in *vrn-3* gene, which (*Vrn-3*) imparts spring habit to wheat (Yan *et al.* 2006; Rousset *et al.* 2011). Vrn-2, the product of *vrn-2* gene (located on 5A chromosome), is the flowering repressor (Yan *et al.* 2004). It keeps *vrn-3* gene repressed until vernalization has been accomplished. Nonfunctional mutations or deletion mutations in *vrn-2* gene also render bread wheat a spring growth habit (Yan *et al.* 2006; Bonnin *et al.* 2008; Distelfeld *et al.* 2009).

Flowering time in winter-wheat and spring-wheat varieties is also controlled by a third class of genes, the *earliness per se* (*eps*) genes. Whereas the major *vrn-1* and *Ppd* genes govern the gross adaptation to environments the *eps* genes have been shown to largely fine-tune the flowering time of wheat varieties for their regional adaptations (Hoogendoorn 1985; Kato and Wada 1999; Snape *et al.* 2001; Hanocq *et al.* 2007; Baga *et al.* 2009; Rousset *et al.* 2011).

It is believed that spontaneous and conscious movement of the active alleles of *VRN1* and *Ppd* genes across landraces and cultivars have been responsible for the worldwide cultivation of bread wheat (Dubcovsky and Dvorak 2007). The *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, *Ppd-B1a* and *Ppd-D1a* alleles singly and in combinations have imparted considerable phenological plasticity to wheat (Pugsley 1971; Stelmakh 1993, 1998; Worland *et al.* 1998; Dubcovsky and Dvorak 2007; Iqbal *et al.* 2007a, b; Mathews *et al.* 2007; Rhone *et al.* 2008; Zhang *et al.* 2008; Eagles *et al.* 2009). Understanding of the regulatory gene network underlying flowering time is a prerequisite for the designing of future wheats for the new and conventional locations and sowing times, especially in the circumstances of climate change (Mathews *et al.* 2007; Rhone *et al.* 2008). The available observations on flowering time and expression of regulatory genes, on certain varieties, single and multiple natural and induced mutants and RNAi constructs against *VRN* and *Ppd* genes grown under a variety of controlled environments, have provided two alternative explanations for the regulation of flowering initiation (Distelfeld *et al.* 2009; Shimada *et al.* 2009). Both of these consider LD (satisfaction of photoperiodic requirement) to be responsible for the upregulation of *VRN-3* (or *TaFT*). Whereas one envisages promotion of *VRN-1* by *VRN-3* (Trevaskis *et al.* 2007; Distelfeld *et al.* 2009), the other proposes that upregulation of *VRN-3* involves both VRN-1 and LD (Shimada *et al.* 2009; Trevaskis 2010).

Some information about the interaction between *VRN-1* and *Ppd* active alleles have been generated from field experiments where wheat germplasm diagnosed for allelic composition at the *VRN-1* and *Ppd-D1* loci had been cultivated in the agroclimates of their adaptation and characterized for flowering time. In fully vernalized winter wheat plants, *Ppd-D1a* advanced flowering by up to 24 days (Gonzalez *et al.*

2005; White *et al.* 2008). The flowering time of wheat carrying an active allele of *VRN-1* was atleast 30 days earlier (Gonzalez *et al.* 2005; Bespalova *et al.* 2010). *Ppd-D1a* in the presence of one or more active *VRN-1* allele(s) advanced flowering time by up to 12 days (White *et al.* 2008; Eagles *et al.* 2010); flowering time was advanced by 11.8 days when *Vrn-A1*, *Vrn-B1* and *Vrn-D1* were present simultaneously and only by 3.7 days when *Vrn-B1* alone was present in addition to *Ppd-D1a* (Eagles *et al.* 2010). In the presence of *Vrn-B1* on one hand and *Vrn-D1* on other hand, the differences between the flowering times of *Ppd-D1a* (photoperiod-insensitive) versus *Ppd-D1b* (photoperiod sensitive) were 3.5 days and 4.9 days, respectively (Eagles *et al.* 2010). The *VRN-1* genotypes were observed to be marginally early in flowering time in the following order *Vrn-A1 Vrn-B1 Vrn-D1* < *Vrn-A1 Vrn-B1*, *Vrn-A1 VrnD1* and *Vrn-A1* < *Vrn-B1* and *Vrn-D1* (Iqbal *et al.* 2007a, b; Eagles *et al.* 2009). These observations indicated epistasis between *VRN-1* active alleles and these and *Ppd-D1a* active allele (Eagles *et al.* 2009, 2010). Overall, at best the results can be taken to mean that *Ppd* and *VRN-1* genes participate in the same pathway of flowering time determination (Distelfeld *et al.* 2009).

For the last few years, we have been investigating the phenology of some Indian wheat cultivars by sowing them in all seasons of calendar year for identifying those that may be suitable for cropping in the autumn season, in addition to the conventional winter season (Kumar 2009). These experiments have identified some cultivars that are very early flowering. The diagnosis of their *VRN-1* and *Ppd-D1* allelic composition showed the super earliness to be a consequence of complementary interaction between the active alleles of *VRN-1* and *Ppd-D1* genes. This inference was confirmed by studying the flowering time in isogenic lines constructed to carry *Ppd-D1b* or *Ppd-D1a* loci in combination with active *VRN-1* loci. The study also revealed the role of temperature in the attainment of flowering competence in the photoperiod and vernalization-insensitive genotypes. The above experiments are reported here.

Material and methods

Plant material

The plant material comprised of 12 spring wheat varieties, released for cultivation at different times and for various agroclimates in India, the Chinese Spring line and several lines developed in the present work. The Indian cultivars were WR544 (Pusa Gold), Lok 1, VL401, GW173, GW496, HD2851 (Pusa Vishesh), Sonalika, K7903 (Halna), HD2687 (Pusa Shresth), NP846, C591 and PBW343. Among these HD2687 and PBW343 often serve as standards in current breeding programmes for developing superior genetic material in spring wheat for the northwest and central Indian plains (and Indo-Gangetic plains) agroclimate(s). The new lines were developed from the cross WR544 × NP846. The early flowering F_2 plant number 44, whose flowering time matched with that of WR544 was developed into the $F_{2-44:6}$ recombinant inbred line by selfing over six generations. The $F_{2-44:3}$ was backcrossed thrice to WR544 on one hand and to NP846 on other hand to evolve the early flowering lines $BC_3F_{2-20:3}$ and $BC_3F_{2-22:3}$, respectively. A late flowering $F_{2-30:3}$ line whose flowering time matched with that of NP846 was backcrossed to NP846 on the one hand and WR544 on other hand to develop $BC_3F_{2-17:3}$ and $BC_3F_{2-13:3}$ lines, respectively. The crosses were advanced rapidly by growing the progenies tandemly. Flowering time behaviours and suitable DNA markers were used to monitor the genetic compositions of the F_1 (WR544 × NP846) plants and allelic structure at the *vrn* and *Ppd-D1* loci in the $F_{2:3}$ and advanced generations.

Cultivation conditions

Plants were grown in clay pots and field plots at the experimental farm of NIPGR, New Delhi, India (28.58 N latitude and 77.20 E longitude). For DNA extraction leaves were used from the pot and field grown plants, labelled and stored at −80°C. To advance the progenies and to measure flowering time, genotypes were grown under field conditions. The soil type in all the plots were free-draining, sandy loam in texture, medium in fertility and slightly alkaline. Land was managed conventionally, using small manual equipments, farm yard manure, synthetic fertilizers and agrochemicals. Before each sowing, plots were applied 50 kg ha^{-1} urea, 20 kg ha^{-1} P_2O_5, 20 kg ha^{-1} K_2O, 10 kg ha^{-1} $ZnSO_4$, 0.2 kg ha^{-1} chloropyrophos and 5 t ha^{-1} farm yard manure. Seeds were sown in 1.5-m long furrows at the depth of about 5 cm. The row to row distance was 0.25 m in 6 m^2 blocks. Fifty seeds were sown per row. For each sowing time three rows of each genotype were sown in two different blocks. Crops were flood irrigated as and when required; and moisture stress was avoided. Weeding was done manually. Seeds of 13 spring wheat cultivars/lines were sown in the middle of each month from 15 April 2005 onwards. Calendar monthwise data on mean air temperature (°C) and day length (h) over the five years period of experimentation from 15 April 2005 is summarized in figure 1. New Delhi experiences five seasons: summer, April–June/July; monsoon rain, July–September; autumn, September–November;

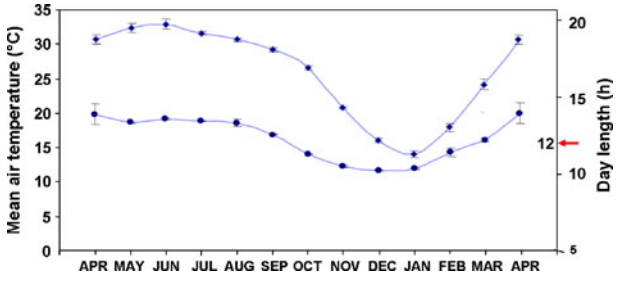

Figure 1. Mean air temperature (♦) and day length (●) for each calendar month experienced over the period 2005–2010, in New Delhi.

Table 1. PCR markers used for determining the presence or absence of the dominant alleles for vernalization-insensitivity (*Vrn-A1, Vrn-B1, Vrn-D1* and *Vrn-3*) and photoperiod-insensitivity *Ppd-D1a*.

Gene and allele	PCR primers (5′ → 3′)	Primer name	Annealing temperature (°C)	PCR product band/marker size (bp)	Reference
Ppd-D1a	F=ACGCCTCCCACTACACTG	Ppd-D1_F	54.0	288	Beales *et al.* (2007)
	R1=GTTGGTTCAAACAGAGAGC	Ppd-D1_R1			
	R2=CACTGGTGGTAGCTGAGATT	Ppd-D1_R2			
Ppd-D1	R2=CACTGGTGGTAGCTGAGATT	Ppd-D1_R2	54.0	414	Beales *et al.* (2007)
Vrn-A1a	F=GAAAGGAAAAATTCTGCTCG	VRN1AF	50.9	965, 876	Yan *et al.* (2004)
	R=GCAGGAAATCGAAATCGAAG	VRN1-INT1R			Zhang *et al.* (2008)
Vrn-A1b	R=GCAGGAAATCGAAATCGAAG	VRN1-INT1R	50.9	714	Zhang *et al.* (2008)
Vrn-A1c	R=GCAGGAAATCGAAATCGAAG	VRN1-INT1R	50.9	734	Zhang *et al.* (2008)
vrn-A1	R=GCAGGAAATCGAAATCGAAG	VRN1-INT1R	50.9	734	Zhang *et al.* (2008)
Vrn-A1c	F=AGCCTCCACGGTTTGAAAGTAA	Intr/A/F2	56.0	1170	Fu *et al.* (2005)
	R=AAGTAAGACAACACGAATGTGAGA	Intr/A/R3			Iqbal *et al.* (2007a,b)
					Zhang *et al.* (2008)
vrn-A1	F=GCACTCCTAACCCACTAACC	Intr1/C/F	58.0	1068	Zhang *et al.* (2008)
	R=TCATCCATCATCAAGGCAAA	Intr1/AB/R			
Vrn-B1	F=CAAGTGGAACGGTTAGGACA	Intr/B/F	58.0	709	Zhang *et al.* (2008)
	R=CTCATGCCAAAAATTGAAGATGA	Intr/B/R3			
Vrn-D1	F=GTTGTCTGCCTCATCAAATCC	Intr/D/F	61.0	1671	Zhang *et al.* (2008)
	R=GGTCACTGGTGGTCTGTGC	Intr/D/R3			
Vrn-3	F=CATAATGCCAAGCCGGTGAGTAC	FT-B-INS-F	57.0	1200	Yan *et al.* (2004)
	R=ATGTCTGCCAATTAGCTAGC	FT-B-INS-R			Zhang *et al.* (2008)
	F=ATGCTTTCGCTTGCCATCC	FT-B-NOINS-F		1140	
	F2=GCTGTGTGATCTTGCTCTCC	FT-B-NOINS-F2		691	
	R=CTATCCCTACCGGCCATTAG	FT-B-NOINS-R			

winter, December–February; and spring, February–March. Spring wheat could be successfully evaluated for flowering time by growing through all the seasons. Although fertility was low in crops that flowered during intense summer and monsoon, yet some seeds that germinated and produced progeny plants were obtained from all the lines in the hot/wet seasons. Thus the progenies from crosses could be rapidly advanced. The date of sowing of progenies from crosses varied, as per the progressive availability of the advanced generation seeds.

Recording of observations

To record the heading date, wheat plots were inspected daily. The heading date of a line was treated as the day on which 50% of the ears had fully emerged from the flag leaf sheath of their first tiller. At this time, the number of leaves formed on the tiller was also recorded. Flowering time was estimated as number of days from the day of sowing to the day of ear emergence. For each of the sowing time, the genotypewise sum of the means of daily maximum and minimum temperatures and sum of day light hours across the period from sowing time to flowering time was respectively taken as the thermal degree days (TDD; Mathews *et al.* 2007) and total day light received by plants before their flowering (TDL; Roberts *et al.* 1986).

PCR assays

The genotypewise DNAs for PCR analyses were isolated from leaves resourced from three-weeks-old seedlings raised in pots or young leaves plucked from field grown plants, using the CTAB method (Saghai-Maroof *et al.* 1984). ISSR primer sets that distinguished WR544 and NP846 were used to ascertain the hybrid nature of F_1 plants. The sequences of the primers used, for testing the presence of various alleles related to flowering time, are given in table 1. The PCR reactions were performed in a MyCycler Personal Thermal Cycler (Bio Rad, Foster City, California, USA).

The PCR reactions with ISSR primers were performed in a 25 μL volume containing 1 U of *Taq* DNA polymerase (Sigma, St Louis, USA), 40 ng template DNA, 0.8 μM of primer, 0.1 mM each of dNTPs (Amersham Biosciences, Uppsala, Sweden), 2.5 μL of 10× PCR reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.4)] and 3 mM $MgCl_2$. The PCR schedule was 1 cycle of 60 s at 94°C, 30 s at 36°C and 60 s at 72°C followed by 45 cycles of 5 s at 94°C, 15 s at 36°C and 60 s at 72°C and a final cycle of 7 min at 72°C; (b) 1 cycle of 2 min at 94°C followed by 35 cycles of 25 s at 94°C, 50 s at 50°C and 90 s at 72°C, and a final cycle of 7 min at 72°C. The products of reactions were resolved on 1.5% agarose gels, with 1× TBE (45 mM Tris-borate and 1 mM EDTA) buffer. The thermocycling conditions for PCR reactions for the detection of *Vrn1* and *Ppd-D1a* alleles were adapted after Sherman *et al.* (2004), Yan *et al.* (2004), Fu *et al.* (2005), and Beales *et al.* (2007). The reactions for

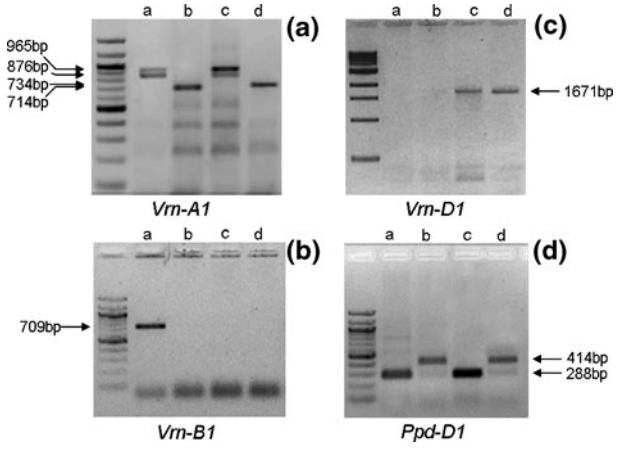

Figure 2. PCR amplification products demonstrating the presence or absence of *Vrn-A1a*, *Vrn-A1b, vrn-A1, Vrn-B1*, *Vrn-D1* and *Ppd-D1* alleles in the selected four genotypes WR544 (a), PBW343 (b), Sonalika (c) and Chinese Spring (d) of bread wheat *Triticum aestivum*. (a) The amplification products of the sizes 965 and 876, 714 and 734 bp primed by the primer pair VRN1AF and VRN1-INT1R for the *Vrn-A1a*, *Vrn-A1b* and v*rn-A1* alleles were respectively seen in the DNA of a and c, b and d. (b) The amplification product of the size 709 bp primed by the primer pair Intr/B/F and Intr/B/R3 for the *Vrn-B1* allele was seen only in the DNA of a (DNA of b, c and d did not amplify the locus). (c) The amplification product of the size 1671 bp primed by the primer pair Intr/D/F and Int/D/R3 for the *Vrn-D1* allele was seen in the DNA of c and d (amplification was not seen with the DNA of a and b). (d) The amplification product of the size 288 bp primed by the forward primer (Ppd-D1_F) and reverse primers Ppd-D1_R1 and Ppd-D1_R2 for the *Ppd-D1a* allele was seen in the DNA of a and c (the fragment with the size 414 bp represents the wildtype allele of the gene in b and d).

Vrn 1 and *Ppd-D1a* diagnostic markers were performed in 20 μL and 25 μL volumes, respectively. The reaction mixture contained 1 U of *Taq* DNA polymerase and 50–100 ng of genomic DNA and 4–5 μM each of the primers, forward primer and one or two reverse primers. The PCR mixtures for the test of *Vrn1* alleles contained 0.08 mM each of the dNTPs, 2 μL of 10× PCR buffer and 2.6 mM $MgCl_2$. After initial denaturation at 94°C for 4 min, 40 cycles were run at 94°C for 30 s, 51–61°C (depending on the primer pair used) for 30 s, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. For the detection of *Ppd-D1* alleles the PCR reaction mixture contained 0.1 mM each of the dNTPs, 2.5 μL of 10× PCR buffer and 3.5 mM $MgCl_2$. Initial denaturation at 94°C for 2 min was followed by 30 cycles of 94°C for 40 s, 54°C for 30 s, 72°C for 1 min. PCR products were visualized on 1.5% and 1.2% agarose for *Vrn1* and *Ppd-D1* alleles, respectively (figure 2).

Results

Relationship between flowering time and genotype at the vrn-1 and Ppd-D1 loci

From among several hundred spring wheat cultivars that have been released for regionwise cultivation in India, 12

representative cultivars were chosen for inclusion in the present study. The sample included both pregreen and post-green revolution cultivars. Included were varieties developed for the conventional wheat cropping season (HD2687 or Pusa Shresth, Lok1, VL401, GW496, PBW343, HD2851 or Pusa Vishesh, NP846 and C591) that is sowing of seeds in second half of November and harvesting of crop in late March / early April, and those suitable for very late sowing in early January (WR544 or Pusa Gold, GW173, K7903 or Halna and Sonalika). The extensively characterized experimental line Chinese Spring was included as the internal control. The panel of the above wheat lines was evaluated for their flowering times under natural environmental conditions at New Delhi. For each of the lines, seeds were sown in the middle of each calendar month from April 2005 to March 2010. The mean values of days to flowering from the date of seed sowing and corresponding thermal degree days and total day length for the flowering times of 13 lines for 12 dates of seed sowing are shown in figure 3. By sowing them every month of the calendar year the behaviour of the tested lines could

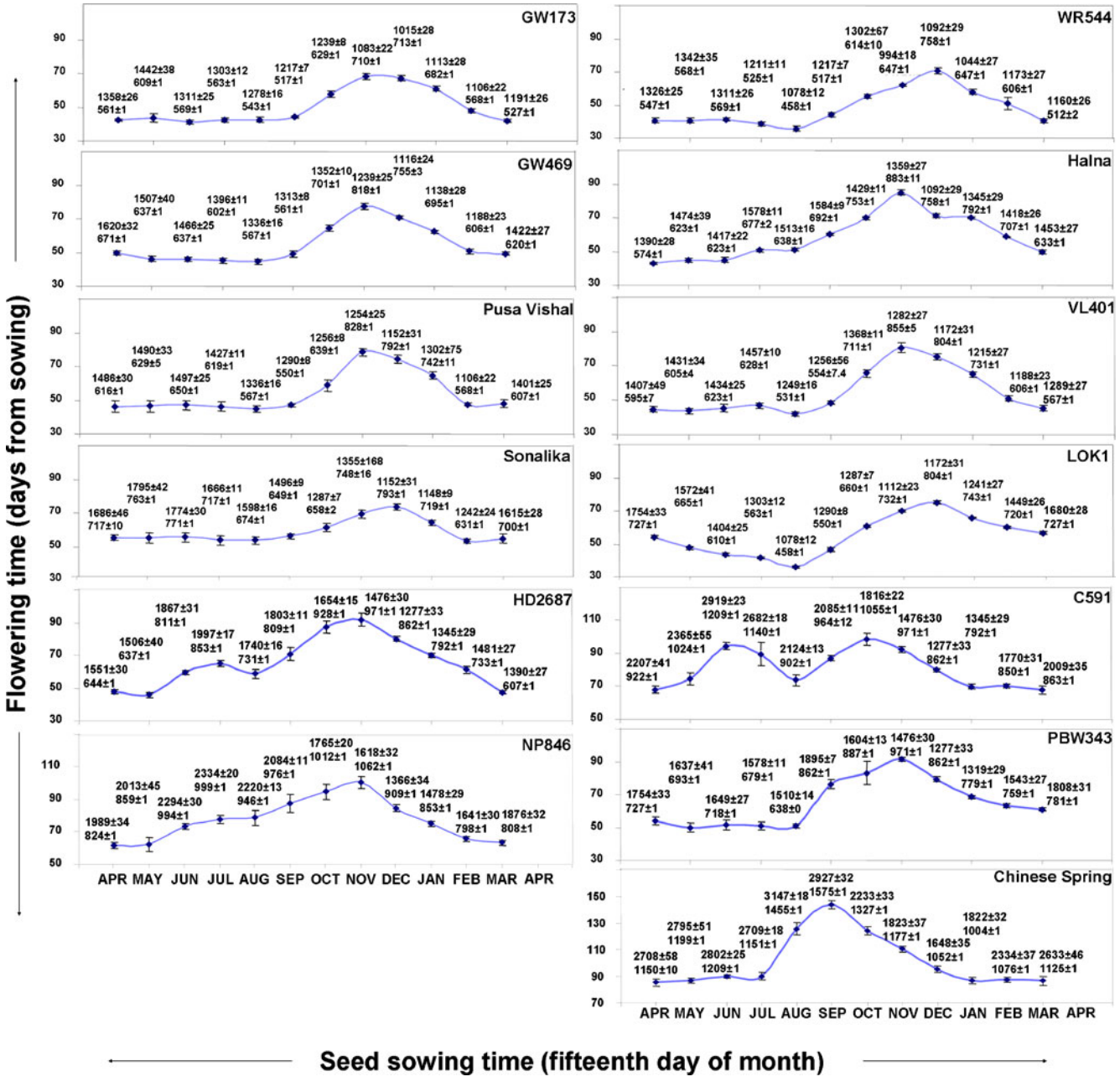

Figure 3. Effect of seasons on the flowering time (d, number of days from seed sowing to heading of principal tiller) of 12 Indian cultivars and the line Chinese Spring of the spring bread wheat *Triticum aestivum*. The seeds were sown in the middle of each month, starting from 15 April 2005 to 15 March 2010. Each flowering time value is average of observations over five years. Given above of each of the flowering time point are the corresponding averages of thermal degree days to flowering (top) and total day length (d) for flowering time (bottom).

be studied under a continuum of environments. From the observations presented in figure 3, the crops of each line that had the shortest and longest flowering times were identified. In concurrence with the day length data (figure 1), for each line the observed shortest and longest flowering times were treated equal to their flowering times under LD (>12 h light) and SD (<12 h light), respectively. Table 2 gives linewise sowing dates of the LD and SD crops as identified above, flowering times of the crops, related day-light hours and thermal degree days and genotype with respect to *VRN-1*, *VRN-3* and *Ppd-D1* loci. The flowering time properties of the wheat lines averaged for all the sowing dates together with their genotype at the *VRN-1* and *Ppd-D1* loci are shown in table 3.

The studied spring wheat lines demonstrated enormous variability in their flowering time response to LD and SD conditions encountered on account of a spectrum of sowing dates (figure 3; table 2). It will be seen from table 2 that in the Indian varieties, the LD flowering time varied from 36 to 68 days and SD flowering time from 68 to 100 days. The flowering times of Chinese Spring were the longest, 85 in LD and 144 in SD. The SD and LD flowering times were highly correlated ($r = +0.98$). When flowering times of the lines were averaged over all the 60 sowing times (table 3), 48.2 ± 3.2 days was the earliest and 100.9 ± 5.8 was the latest flowering time.

The results of screening for the presence or absence of five *Vrn-1* (*Vrn-A1a, Vrn-A1b, Vrn-A1c, Vrn-B1* and *Vrn-D1*), a *Vrn-3* and *Ppd-D1a* alleles are given in the table 2. All the genotypes studied were found to possess one or more of the *Vrn-1* allele(s). Several Indian varieties were observed to carry all of the *Vrn-1* alleles, *Vrn-A1*, *Vrn-B1* and *Vrn-D1*. The *Vrn-A1a* allele was found to be present in all the Indian varieties except PBW343. In PBW343 the only *Vrn-1* allele present was *Vrn-A1b*. The present observations confirmed the earlier finding that the presence of only one *Vrn-1* mutation in the genome is sufficient for acquirement of spring growth habit in the wheat *T. aestivum* (Fu *et al.* 2005; Trevaskis *et al.* 2007; Shimada *et al.* 2009; Trevaskis 2010). None of the genotypes were observed to carry the *cis*-dominant *Vrn-3* allele that was tested. Therefore, it is further concluded that the concerned mutation did not contribute to spring habit of the Indian varieties studied here.

The genotypes fell into two distinct groups on the basis of flowering time under SD conditions (table 2) and all the sowing regimes (table 3). Eight lines, namely WR544, Lok1, VL401, GW173, GW496, HD2851, K7903 and Sonalika, comprised an early flowering group and five lines (HD2687, NP846, C591, PBW343 and Chinese Spring) formed a late flowering group. Under SD conditions, the early flowering group flowered in ≤85 (mean 76.3 ± 2.0) days and the late flowering group flowered in ≥92 (mean 105.4 ± 9.8) days (the two groups are statistically different, $t_{11} = 3.67$, $P < 0.01$). Taking into consideration all the sowings, the mean flowering times of the early and late flowering groups were 54.2 ± 3.1 (range 48.2 ± 3.2–58.9 ± 2.0) and 77.7 ± 3.6 (range 65.1 ± 4.1–100.9 ± 5.8) days, the 23.5 days difference is highly statistically significant ($t_{154} = 9.41$, $P < 0.01$). Importantly, the *Ppd-D1a* allele was present only in the members of the early flowering group. These observations allowed the conclusion that additive/complementary interaction between *Vrn-1* and *Ppd-D1a* mutations leads to earliness in spring bread wheat or super earliness in bread wheat, both under LD and SD conditions.

Interaction between vernalization-insensitivity and photoperiod-insensitivity

The interaction between *VRN-1* and *Ppd-D1* genes was also studied by comparing the flowering times of *Vrn-1 Ppd-D1b* and *Vrn-1 Ppd-D1a* genotypes constructed by use of the backcrossing technique in the genetic backgrounds of WR544 and NP846. The results (table 4) showed that flowering times of WR544, $BC_3F_{2-20:3}$ (in WR544 background) and $BC_3F_{2-22:3}$ (in NP846 background), all those having active *Vrn-1* and *Ppd-D1a* alleles were respectively 38, 40 and 46 days in autumn sown conditions and 69, 72 and 73 days in winter sown conditions. The flowering times of NP846, $BC_3F_{2-13:3}$ (in WR544 background) and $BC_3F_{2-17:3}$ in NP846 background) were respectively 88, 87, and 87 days in autumn sown conditions and 83, 83 and 84 days in winter sown conditions. The flowering times of autumn and winter sown crops of Chinese Spring were 160 and 93 days, respectively. These results confirmed that the interaction between vernalization-insensitivity and photoperiod-insensitivity is complementary. The expression of the interactive effect is affected by cropping conditions; it is highly manifested in crops sown in LD conditions.

Epistatic interactions between Vrn-1 alleles

Vrn-1 Ppd-D1a wheat lines were genotypically of three kinds at their *Vrn-1* loci: WR544, Lok 1 and VL401, *Vrn-A1a Vrn-B1*; GW173, GW496 and HD2851, *Vrn-A1a Vrn-B1 Vrn-D1*; and K7903 and Sonalika, *Vrn-A1a Vrn-D1*. The average flowering times of the WR544, GW173 and K7903 groups were 52.5, 52.9 and 58.6 days, respectively (table 3). About six days of difference between the flowering times of WR544 and K7903 groups was statistically significant ($t_{58} = 2.0$, $P = 0.05$). This means that the presence of *Vrn-D1* in the absence of *Vrn-B1* was epistatic to *Vrn-A1*. The epistatic interactions between *Vrn-1* alleles have been reported earlier (Iqbal *et al.* 2007a,b; Eagles *et al.* 2010).

Dependence of flowering time on the experienced thermal degree days and total day length

The flowering times of the 13 wheat lines were highly correlated with the corresponding parameters, thermal degree days ($r = 0.56$, $P = 0.003$) and total day length ($r = 0.96$, $P = 0.000$) estimated for LD and SD conditions (table 2)

Table 2. Flowering time features and allelic structure at the *vrn-1*, *vrn-3* and *Ppd-D1* loci in 12 cultivars bred and released for cropping in India and in the landrace Chinese Spring of bread wheat *Triticum aestivum*.

	Season of long days				Season of short days									
									Presence (P) or absence (A) of the specified allele					
									Dominant *Vrn-1* allele[c]				Dominant *Vrn-3*	*Ppd-D1a*
Variety/line	Seed sowing time	First ear emergence time/flowering time (d)	Thermal degree days to flowering time (TDD)	Total day length accumulated until flowering time (TDL) (h:min)	Seed sowing time	Flowering time (d)	TDD	TDL (h:min)	*Vrn-A1a*	*Vrn-A1b*	*Vrn-B1*	*Vrn-D1*	alllele	allele
WR544	15 August	36±1	1078±12 (29.9)[a]	458:14±0.3 (13:13)[b]	15 December	71±1	1092±29 (15.4)	758:30±0.4 (11:08)	P	A	P	A	A	P
Lok1	-do-	37±1	1078±12 (29.1)	458:26±0.2 (12:39)	-do-	75±1	1172±31 (15.6)	804:18±0.3 (11:13)	P	A	P	A	A	P
VL401	-do-	42±2	1249±16 (29.7)	531:35±0.2 (13:05)	15 November	81±3	1282±27 (15.8)	855:14±4.6 (10:56)	P	A	P	A	A	P
GW173	15 April	42±2	1358±26 (32.3)	561:20±0.2 (13:37)	-do-	68±2	1083±22 (15.9)	710:21±0.2 (10:44)	P	A	P	P	A	P
K7903	-do-	43±2	1390±28 (32.3)	574:22±0.2 (13:36)	-do-	85±2	1359±27 (16.0)	883:30±0 (10.39)	P	A	A	P	A	P
GW496	15 August	45±2	1336±16 (29.8)	567:36±0.1 (12:59)	-do-	78±2	1239±25 (15.9)	818:10±0.2 (10.49)	P	A	P	P	A	P
HD2851	-do-	45±2	1336±16 (29.7)	567:30±0.1 (13:01)	-do-	79±2	1254±25 (15.9)	828:36±0.2 (10:48)	P	A	P	P	A	P
HD2687	15 April	48±2	1551±30 (32.3)	644:10±0.2 (13:42)	-do-	92±4	1476±30 (16.0)	971:28±0.3 (10:55)	P	A	P	P	A	A
PBW343	15 August	51±3	1510±14 (29.8)	638:25±0 (12:52)	-do-	92±2	1476±30 (16.0)	971:28±0.4 (10:57)	A[d]	P	A	A	A	A
Sonalika	-do-	54±2	1598±16 (29.6)	674:19±0.6 (12:49)	15 December	73±2	1152±31 (15.8)	793:28±0.3 (11:27)	P	A	A	P	A	P
NP846	15 April	61±2	1989±34 (32.6)	824:25±0.3 (13:52)	15 November	100±2	1618±32 (16.2)	1062:20±0.3 (11:02)	P	A	P	P	A	A
C591	-do-	68±2	2207±41 (32.5)	922:01±0.3 (13:56)	15 October	99±4	1816±22 (18.3)	1055:22±0.1 (11:06)	P	A	P	P	A	A
Chinese Spring	-do-	85±3	2708±58 (31.9)	1150:22±9.6 (13:53)	15 September	144±3	2927±32 (20.3)	1575:15±0.1 (11:34)	A	A	A	P	A	A
Mean of *Vrn-1 Ppd-D1a* lines (rows 1–7 and 10)		43±2				76±2								
Mean of *Vrn-1 Ppd-D1* lines (rows 8, 9 and 11–13)		63±7				105±10								
Mean of *Vrn-1 Ppd-D1a* lines, irrespective of season						60±17								
Mean of *Vrn-1 Ppd-D1* lines, irrespective of season						84±21								
t_{24}[e] between means at 16 and 17						2.68** (0.01)								

[a], Day temperature from the sowing time to flowering time/ear emergence time; [b], mean day length from the sowing time to flowering time; [c], *Vrn-A1c* was absent in all genotypes; [d], A or absence of dominant allele means that counterpart recessive allele was present; [e], *t* test values with degrees of freedom; ** significant at 1% level.

Table 3. Genotypewise flowering time characteristics averaged for crops of 13 cultivars/landrace(s) sown on 15th day of each month over a five year period from April 2005 to March 2010.

Cultivar/landrace	Genotype: Presence (P) or absence (A) of the Dominant *Vrn-1* allele: *Vrn-A1a*	*Vrn-A1b*	*Vrn-B1*	*Vrn-D1*	Semidominant *Ppd-D1a allele*	Mean (±SE) of crops sown on 15th of every month in the years 2005 to 2010[a]: First ear emergence time/flowering time[f] (d)	Total day length to flowering time (TDD)	Thermal degree days accumulated until flowering time (TDL) (h)
WR544[b]	P	A	P	A	P	48.2±3.2	1187.5±34.1	580.6±23.1
Lok1[b]	P	A	P	A	P	54.9±3.5	1361.7±62.6	663.3±28.9
VL401[b]	P	A	P	A	P	54.4±3.9	1312.3±29.6	650.9±29.5
GW173[b]	P	A	P	P	P	50.0±3.0	1221.5±36.3	599.3±20.0
GW469[b]	P	A	P	P	P	54.6±3.2	1340.9±44.1	655.8±22.1
HD2851[b]	P	A	P	P	P	54.1±3.5	1333.1±37.7	650.6±25.9
K7903[b]	P	A	A	P	P	58.3±3.8	1420.9±37.2	696.1±25.3
Sonalika[b]	P	A	A	P	P	58.9±2.0	1484.4±68.6	711.7±14.8
HD2687[b]	P	A	P	P	A	65.5±4.4	1590.6±64.3	781.5±33.2
NP846[b]	P	A	P	P	A	76.8±3.7	1889.6±92.4	920.1±26.0
C591[c]	P	A	P	P	A	80.3±3.3	2006.3±146.1	962.8±36.1
PBW343[b]	A[d]	P	A	A	A	65.1±4.2	1587.5±52.7	779.7±28.4
Chinese Spring	A	A	A	P	A	100.9±5.8	2465.0±140.7	1208.3±48.3
Mean of: WR544, Lok1 and VL401						52.5 ± 3.4	1287.2 ± 36.2	631.6 ± 24.7
Mean of: GW173, GW469, and HD2851						52.9 ± 3.2	1298.5 ± 37.6	635.2 ± 22.1
Mean of: K7903 and Sonalika						58.6 ± 2.8	1452.6 ± 47.6	703.9 ± 15.1
Mean of: All the above cultivars						54.2 ± 3.1	1332.8 ± 36.5	651.0 ± 20.6
Mean of: HD2687, NP846 and C591						74.2 ± 3.5	1828.8 ± 97.0	888.2 ± 26.7
Mean of: PBW343 and Chinese Spring						83.0 ± 4.3	2026.3 ± 91.1	994.0 ± 28.1
Mean of: HD2687, NP846, C591, PBW343 and Chinese Spring						77.7 ± 3.6	1907.8 ± 90.5	930.5 ± 23.2
Mean of: All cultivars/lines						63.2 ± 3.0	1553.9 ± 54.8	758.5 ± 15.9
t_{70}[e] between means at 14 and 15						0.14 ($P = 0.89$)	0.31 ($P = 0.76$)	0.17 ($P = 0.87$)
t_{58} between means at 14 and 16						2.0* ($P = 0.05$)	3.56** (0.0008)	3.09 (0.003)
t_{58} between means at 18 and 19						1.73 ($P = 0.09$)	1.57 ($P = 0.12$)	2.09 (0.04)*
t_{154} between means at 17 and 20						9.41** ($P = 0.0001$)	10.59** ($P = 0.0001$)	12.01** ($P = 0.0001$)

[a], Based on observations on monthly sown crops diagrammed in figure 2; [b], postgreen revolution varieties; [c], pregreen revolution variety; [d], A or absence of dominant allele means that counterpart recessive allele was present; [e], *t* test values with degrees of freedom; [f], it was observed that the total number of leaves formed on the principal tillar in all the 780 enumerations was 5 or 6. *Difference significant at 5%; **difference significant at >1%.

Table 4. Flowering time features and allelic structure at the *vrn-1*, *vrn-3* and *Ppd-D1a* loci in the Chinese Spring line, WR544 and NP846 cultivars, and an early recombinant inbred line, a late and an early line in the background of NP846 and early and late line in the background of WR544, derived from the WR544 × NP846 cross in *Triticum aestivum*.

Genotype						
	Allelic status					
	Dominant *Vrn-1* allele[a]			*Ppd-D1a*		First ear emergence time/
Designation	*Vrn-A1a*	*Vrn-B1*	*Vrn-D1*	allele	Date of seed sowing	flowering time (D)
Chinese Spring	A[b]	A	P	A	8 September 2010	160
					24 December 2010	93
NP846	P	P	P	A	8 September 2010	88
					24 December 2010	83
WR544	P	P	A	P	8 September 2010	38
					24 December 2010	69
WR544 × NP846 = $F_{2-44:3}$, early; $F_{2-44:3}$ = $F_{2-44:6}$, early	P	P	A	P	8 September 2010	42
					24 December 2010	74
WR544 × NP846 = $F_{2-44:3}$, early; $F_{2-44:3}$ × WR544 = $BC_3F_{2-20:3}$, early	P	P	A	P	8 September 2010	40
					24 December 2010	72
WR544 × NP846 = $F_{2-30:3}$, late; $F_{2-44:3}$ × WR544 = $BC_3F_{2-13:3}$, late	P	P	A	A	8 September 2010	87
					24 December 2010	83
WR544 × NP846 = $F_{2-44:3}$, early; $F_{2-44:3}$ × NP846 = $BC_3F_{2-22:3}$, early	P	P	P	P	8 September 2010	46
					24 December 2010	73
WR544 × NP846 = $F_{2-30:3}$, late; $F_{2-30:3}$ × NP846 = $BC_{3-}F_{2-17:3}$, late	P	P	P	A	8 September 2010	87
					24 December 2010	84
Mean of *Vrn-1 Ppd-D1a* genotypes (rows 5 and 7)					8 September 2010	43.0±3
					24 December 2010	72.5±1
Mean of *Vrn-1 Ppd-D1* genotypes (rows 6 and 8)					8 September 2010	87.0±0
					24 December 2010	83.5±1
Grand mean of *Vrn-1 Ppd-D1a* genotypes						57.8±15
Grand mean of *Vrn-1 Ppd-D1* genotypes						85.3±2
t_6[c] between means at 11 and 12						3.17* (0.02)

[a], The dominant forms of *Vrn-A1b*, *Vrn-A1c* and *Vrn-3* were absent from all the genotypes listed here; [b], A or absence of dominant allele means that counterpart recessive allele was present; [c], *t*-test values with degrees of freedom; * significant at 5% level.

and when averaged for all the seasons (table 3), the corresponding correlations were $r = 1.00$, $P < 0.0001$ for thermal degree days and $r = 1.00$, $P < 0.0001$ for total day length. It was further inferred from table 3 that the average air temperature (TDD/flowering time) and mean day temperature (TDL/flowering time) for all the wheat lines approximated 25°C and 12 h respectively. The embryonic flowering, i.e. flowering after emergence of four leaves was not observed in any of the wheat lines in any of the 60 seasons. The number of leaves formed on the first tiller, including the flag leaf, was five or six in all 780 observations.

Variation in flowering time not explained by Vrn-1 and Ppd-D1 alleles

Some of the genotypes that shared the genetic structure at the *Vrn-1* and *Ppd-D1* genes, however differed in their flowering times in SD as well as LD conditions, for example K7903 versus Sonalika and HD2687 versus C591. Such differences among varieties could perhaps be attributed to differences in their *eps* alleles. Thus, it is possible to conclude that the set of Indian varieties included in the study altogether do possess considerable flowering time variation (36 to 68 days in LD conditions and 68 to 100 days in SD conditions) that is determined by a variety of *Vrn-1* alleles, *Ppd-D1a* allele and perhaps *eps* alleles, in various combinations.

Effect of photoperiod on the flowering time in Ppd-D1a spring wheats

The difference between SD flowering time and LD flowering time was unexpectedly large among the photoperiod-insensitive and vernalization-insensitive (*Vrn-1 Ppd-D1a*) genotypes (19 to 42 days; mean 33.3 ± 2.6 days). Shortfall of LD in the short-day predominant season could not be the reason for the above difference, since *Ppd-D1a* mutation would have removed the need of LD for flowering. In the wheat plant, the principal tiller has been observed in flowers only after it has produced five or more leaves, when it is no longer juvenile and could better meet the energy requirements of seed production. Wheat plant growth has been also observed to be highly sensitive to ambient temperature (Barnabas *et al.* 2008; Pancholi *et al.* 2010). The TDD for the SD and LD flowering times were similar for the concerned *Vrn-1 Ppd-D1a* genotypes (table 2). It emerged that the flowering of *Vrn-1 Ppd-D1a* plants is relatively late in SD because a longer span is required to accumulate the critical TDD. Or, it takes longer for plants to come to flower in SD season(s) not only because of the shortage of daylight hours but also because temperatures are lower. These observations led to the conclusion that flowering time is the outcome of bread wheat plant's response not only to vernalization and photoperiod but also to ambient temperature.

Discussion

In the present work 12 cultivars of bread wheat bred in India and the landrace Chinese Spring were sown in the beginning of second week of every month for five years and their flowering times were thus evaluated in a variety of seasons. The 13 wheat lines were genotyped for allelic structure at the *vrn-1*, *vrn-3* and *Ppd-D1* genes/loci. Near isogenic lines of *Vrn-1 Ppd-D1* and *Vrn-1 Ppd-D1a* genotypes were constructed in two distinct genetic backgrounds and evaluated for flowering time under two different sowing times. The study has allowed several important conclusions. (i) It has confirmed that the nature of interaction between the active alleles *Vrn-A1a* and *Vrn-D1* is epistatic; (ii) the interaction for flowering time between *Vrn-1* allele(s) and *Ppd-D1a* is additive/complementary. Although *Vrn-1 Ppd-D1a* genotypes are early flowering under both SD and LD conditions, the flowering time is delayed by low temperature in SD conditions and hastened by high temperature under LD conditions. The *Vrn-1 Ppd-D1a* genotypes demonstrate super-early phenotype under LD conditions; and (iii) the 25°C temperature and 12 h photoperiod perhaps comprise the ambient conditions for flowering. Some aspects of these results are discussed below.

Allele structure at Vrn-1 and Ppd-D1 loci in Indian wheat cultivars

The major areas of wheat cropping in India are in the semitemperate northwestern, central and peninsular plains and northern and southern hills. In northwest and central plains (Indo-Gangetic plains) region, wheat season comprises of the period including late autumn, winter, spring and early summer from November of a year to April of the subsequent year. Wheat crop usually follows summer sown rice or autumn planted potato crops. The crop cycle is of about 150 days for wheat seeded at the optimum sowing time in second half of November and is much shorter when sown late, in January. Among the small sample of 12 Indian varieties included in this study, eight (Lok 1, VL401, GW496, NP2851, HD2687, PBW343, NP846 and C591) had been released for the normal seeding time and four (WR544, GW173, K7903 and Sonalika) for the late sowing times. All the varieties were observed to possess one or more active alleles of *vrn-1* genes and the active allele of *Ppd-D1* gene in five different combinations: *Vrn-A1b*, *Vrn-A1a Vrn-D1*, *Vrn-A1a Vrn-B1 Vrn-D1*, *Vrn-A1a Vrn-B1 Ppd-D1a* and *Vrn-A1a Vrn-B1 Vrn-D1 Ppd-D1a*. All the varieties had an *Vrn-A1* allele. PBW343 contained only one *Vrn-1* allele the *Vrn-A1b* gene. This observation means that presence of only one active *Vrn-1* gene is sufficient for the acquirement of spring habit under the mild winter conditions of Indian wheat cultivation environment. Several varieties from among those recommended for timely sowings and all recommended for late sowings carried the *Ppd-D1a* gene. The *Vrn-B1* and/or *Vrn-D1* active alleles were found to be present

both in absence of and together with *Ppd-D1a*, in varieties recommended for timely sowings. *Vrn-B1* and/or *Vrn-D1* alleles were observed to be invariably present in varieties recommended for late sowings. The only pregreen revolution variety included in the study C591 possessed *Vrn-A1a Vrn-B1 Vrn-D1* genotype. Indian landraces were the source of *Vrn-D1* for early flowering in the early Australian varieties (Eagles *et al*. 2010). *Vrn-D1* in Indian varieties has most likely moved from Indian wheat germplasm. The presence of *Vrn-A1* and *Vrn-B1* alleles in C591 indicates their introgression from the spring wheat germplasm used in the construction of varieties grown in north American high latitudes (Paterson *et al.* 2000; Iqbal *et al.* 2007a, b). The incorporation of *Ppd-D1a* allele in the postgreen revolution varieties is related to the use of germplasm gained from CYMMIT (International Maize and Wheat Improvement Centre; Borlaug 1983). Genotyping at *vrn* and *Ppd* loci in a small sample of 12 Indian wheat cultivars indicates that the Indian germplasm does harbour critical mutations in the *vrn* and *Ppd* genes for breeding new varieties of wheat adapted to the new and emerging microagroclimatic conditions in the geographical areas of wheat cultivation (Asseng *et al.* 2011).

Role of Vrn-1 in the determination of flowering time

Knowledge about the mode of action of the network of *vrn-1*, *vrn-2* and *vrn-3* gene products in the determination of flowering time of bread wheat (Colasanti and Coneva 2009; Distelfeld *et al.* 2009; Greenup *et al.* 2009; Jung and Muller 2009; Trevaskis *et al.* 2010) has been advanced by an observation made in the present study. Among the products of *vrn* genes, Vrn-2 is known to repress flowering by repressing the expression of *vrn-1* and *vrn-3* genes (Yan *et al.* 2004; Distelfeld *et al.* 2009). Vrn-3 has been identified as the florigen synthesized in the LD which upregulates the *vrn-1* expression and downregulates *vrn-2* expression (Yan *et al.* 2006; Trevaskis *et al.* 2007; Li and Dubcovsky 2008). Vrn-1 induced by vernalization has been shown to be essential for flowering because it is a regulator of genes involved in the transitioning of apical meristem from vegetative to reproductive (flowering) phase (Shitsukawa *et al.* 2007; Distelfeld *et al.* 2009; Shimada *et al.* 2009; Allard *et al.* 2012). It has also been established earlier that because of the *cis*-dominant mutation Vrn-1 is constitutively synthesized in the spring wheats (Yan *et al.* 2004; Fu *et al.* 2005; Loukoianov *et al.* 2005; Pidal *et al.* 2009). Vrn-1 is also known to be synthesized both in leaves and tiller apical meristem (Loukoianov *et al.* 2005). The embryos in wheat seeds have been shown to carry primordia for up to 3 or 4 leaves only (Kirby and Appleyard 1987). Since in the present study, tests on all crops, of all genotypes, taken in different seasons throughout calendar year, did not show embryonic flowering in the principal tiller and the principal tiller formed its ear only after producing five or more leaves, the involvement of Vrn-1 in the promotion of vegetative growth in juvenile stage plants via its role in enhancement of meristematic activity in the leaf primordia and apical meristem is indicated.

Nature of interaction between Vrn-1 and Ppd-D1 genes

The principal finding of this study is that the nature of interaction between the vernalization-insensitive (*Vrn-1*) mutant alleles of *vrn-1* genes and photoperiod-insensitive (*Ppd-D1a*) mutant allele of *Ppd-D1* gene is of complementary/additive type. In recent years, precise quantitation of such interactive effect(s) has been facilitated by the development of molecular markers for the detection of various *vrn-1* and *Ppd-D1* alleles. Flowering time enumeration in the vernalized crops of winter wheat varieties on the one hand and isogenic lines of winter wheat on the other hand showed that the *Ppd-D1a* advanced flowering time by 11.2 days (White *et al.* 2008) and 23/24 days (Gonzalez *et al.* 2005), respectively. Corresponding comparisons between *Ppd-D1a* and *Ppd-D1* spring wheat varieties and isogenic lines showed that *Ppd-D1a* hastened flowering only by 5.2 days (Eagles *et al.* 2009) and 3.1 days (Dyck *et al.* 2004), respectively. In these studies the observed advance in flowering time was much less than expected for full additive interaction between *Vrn-1* and *Ppd-D1a* active alleles. The results reported in tables 2, 3 and 4 of present study provide clear evidence for additivity in the *Vrn-1* × *Ppd-D1a* interaction(s). The average difference between the flowering times of *Vrn-1 Ppd-D1a* and *Vrn-1 Ppd-D1* wheat lines was −29.2 days under SD conditions and −19.6 under LD conditions (table 2). Under all the 60 seasons considered together the flowering in *Vrn-1 Ppd-D1* was late by 23.5 days as compared to *Vrn-1 Ppd-D1a* varieties (table 3). Under two dates of seed sowing, the *Vrn-1 Ppd-D1a* lines flowered 27.5 days earlier than in the isogenic *Vrn-1 Ppd-D1* lines (table 4). Observations of additivity in the interaction between *Vrn-1* and *Ppd-D1a* mutations support the idea that *Vrn-1* and *Ppd-D1a* are in separate branches, in the pathway of transition from vegetative phase to reproductive phase, which convergently upregulate *Vrn-3* (*TaFT*) expression to synthesize the mobile floral promoter (Trevaskis 2010). Additional information about the mechanism of interaction between *Vrn-1* and *Ppd-D1a* was provided by the following observation. The genotypes of wheat that carried one or more *Vrn-1 cis*-dominant allele(s) together with *Ppd-D1a* allele flowered earlier in LD season than in SD season (table 2). Since in these genotypes the requirement of LD for flowering had been overcome by the presence of *Ppd-D1a* mutation, the delay in flowering in SD must be related to slower rate of their growth in SD season. Winter months are a major component of SD season under the New Delhi agroclimate conditions. The flowering delay in *Vrn-1 Ppd-D1a* mutants in SD conditions was thus observed to be correlated with the developmental lag undergone by the juvenile plants in the colder SD season. Accordingly, it is inferred that the *Ppd-D1* expression is not only controlled by LD but also by the plant's developmental

status. Whereas the *Ppd-D1a* mutation nullified the requirement of LD for flowering, the need for attainment of the optimum developmental state for the *Ppd-D1a* expression remained unfulfilled. It is suggested that there is an intermediate state of plant development, between juvenile and reproductive stage, which is critical for the *Ppd-D1* gene expression. Once a wheat tiller reaches this hypothesized developmental state, Ppd-D1 directed flowering pathway becomes functional. Vrn-3 is synthesized and it is presumably transported to the tiller apical meristem for the activation of *Vrn-1* gene via the Vrn-3 signal. The present results suggest that the complementary action between *Vrn-1* and *Ppd-D1a* in spring bread wheat means the following. The expression of vernalization independence *Vrn-1* gene provides Vrn-1 function for vegetative growth in the principal tiller for it to grow to a developmental stage critical for the expression of the photoperiod independence *Ppd-D1a* gene. The availability of Ppd-D1a then induces Vrn-3 synthesis in leaves. In conformity with the already presented idea (Trevaskis 2010), Vrn-3 transported from leaves to the tiller apex then provides for the hyperinduction of *Vrn-1* gene. The Vrn-1 then turns on the spike and floret developmental pathways.

Concluding remarks

The study of flowering time biology in bread wheat, briefly discussed above, has led to the following general conclusion. The temperature and photoperiod effects on the expression of vernalization responsive, photoperiod responsive and earliness *per se* genes, from the time of seed sowing/germination to flowering, control the flowering time of a wheat line. A priori it can be suggested that, under the natural field conditions of growth, the intrinsic genetic contribution to flowering time phenotype is equal to the mean of flowering time phenotypes estimated under a large number of the possible combinations of temperature and photoperiod. For each wheat line studied here, there were 60 flowering time observations and for each observation the cumulative thermal degree days and total day length parameters had been estimated. Interestingly, the average air temperature and day length over the flowering time period for each of the 13 wheat lines was found to be about 25°C (24.6 $\pm$ 0.08) and 12 h (12 $\pm$ 0.01). This analysis led to two inferences. (i) For the attainment of flowering in spring wheat, 25°C air temperature and 12 h photoperiod are the ambient environmental conditions; and (ii) under these conditions of growth, the flowering time phenotype manifests the genetic contribution.

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