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Molecular characterization of vernalization genes in Iranian wheat landraces

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ABSTRACT

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Wheat, a globally important food crop, is widely grown in various regions of the world. Wheat's wide adaptation to different climatic conditions is strongly controlled by vernalization (*Vrn*), photoperiod (*Ppd*) and earliness *per se* (*Eps*) genes. In this study, 395 Iranian wheat landraces were characterized by applying markers for the vernalization genes *Vrn-B1*, *Vrn-D1*, and *Vrn-B3*. Based on specific primers of the recessive *vrn-B3* allele, two novel fragments in addition to the expected alleles were amplified. Spring habit *Vrn-D1* and *Vrn-B1* alleles were observed in 67.35% and 38.48%, respectively, of Iranian wheat landraces. Twenty-nine landraces were heterozygous for the *Vrn-D1* gene. The growth habit of the test wheat landraces could not be completely predicted by their allelic status at the *Vrn-1* genes. This inconsistency may be due to misclassification of the growth habit of the studied landraces, the presence of a new mutation at *VRN* loci in Iranian landraces or other functional genes such as *Ppd* and *Eps* genes which were not included in this survey. Therefore, sequencing the putative alleles at various *VRN* loci of spring and winter types could provide useful information.

Keywords: growth habit, promoter region, retrotransposon element, wheat landraces

INTRODUCTION

Wheat landraces undoubtedly represent an important source of genetic diversity that could be used for wheat improvement in future (Dreisigacker *et al*., 2005; Akar and Özgen, 2007). The rich diversity of wheat landraces from Iran has not been sufficiently analyzed from a genetic point of view. Iran is one of the primary habitats of wheat's ancestors. Landraces possess many important traits due to longterm adaptation to their native environment. They also have the advantage of being easily incorporated into breeding programs and, compared with wheat's wild relatives, they are easy to cross with elite breeding materials. For these reasons, more information about the genetic diversity, adaptability and relationships among landraces would be invaluable for utilizing existing genetic resources (Zhang *et al*., 2006).

Many temperate cereals, including wheat and barley, require a period of cold temperature treatment to switch from the vegetative to the reproductive phase (Sun *et al*., 2009). This is called the "vernalization requirement." The growth habit of wheat is largely governed by three groups of genes including vernalization (*Vrn*) genes, photoperiod (*Ppd*) genes, and genes controlling earliness *per se* (*Eps*) (Kato and Yamagata, 1988). These groups act together to determine the timing of flowering and wheat's adaptation to a particular environment (Worland, 1996; Worland *et al*., 1998). Based on the response to the vernalization requirement, wheat is divided into winter and spring types. Different frequencies of *Vrn* alleles have been reported from different parts of the world, suggesting that these allele combinations play an important role in wheat's adaptation to different climatic conditions (Stelmakh, 1990, 1998; Goncharov, 1998; Iwaki *et al*., 2000, 2001).

Studies have shown that the vernalization requirement in commercial wheat cultivars is controlled by at least five loci; *VRN-A1*, *VRN-B1*, *VRN-D1*, *VRN-4*, and *VRN-B3* (Pugsley, 1971, 1972; Goncharov, 2003; Yan *et al*., 2006). Recently a new *VRN-B3* allele was identified in cultivar Hope, which is located on the short arm of chromosome 7B (Yan *et al*., 2006). The *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, and *Vrn-B3* genes are dominant for spring growth habit and epistatic to the alleles for winter growth habit (Pugsley, 1971). Winter cultivars have recessive alleles at the three *VRN-1* loci (Stelmakh,

1987), whereas spring cultivars have dominant alleles at one or more of these genes. The dominant *Vrn* alleles exhibit differential sensitivity to vernalization. For example, dominant *Vrn-B1*, *Vrn-D1* or *Vrn-D4* cause a low vernalization requirement, whereas dominant *Vrn-A1* causes insensitivity to vernalization (Shindo and Sasakuma, 2002) and masks the low vernalization requirement of other dominant alleles in wheat.

Variation in dominant alleles of vernalization genes causes differences in the timing of flowering, formation of yield components, and plant height in wheat cultivars (Stelmakh, 1993; 1998; Gororo *et al*., 2001). Stelmakh, (1993) reported that average grain yield was highest for spring wheat genotypes having *Vrn-A1* and/or *Vrn-B1* alleles, whereas cultivars with triple dominant alleles (*Vrn-A1*, *Vrn-B1*, *Vrn-D1*) had the lowest grain yields. Iqbal *et al*. (2011) concluded that a combination of dominant *Vrn-A1a*, *Vrn-B1*, and *Vrn-D1* alleles results in wheat cultivars with the earliest maturity but that are low yielding.

The dominant *Vrn-B1* and *Vrn-D1* alleles contain large deletion in intron-1 of tetraploid and hexaploid wheat (Yan *et al*., 2004b; Fu *et al*., 2005), whereas dominant *Vrn-B3* possesses a retrotransposon element insertion in the promoter region (Yan *et al*., 2006). Santra *et al*. (2009) identified novel allele *Vrn-B1b* in wheat cultivar Alpowa, which was 673-bp in length. Chu *et al*. (2011) reported another novel *Vrn-B1* allele with a 5463-bp retrotransposon insertion that was in 5′-UTR of the promoter region and caused spring growth habit in tetraploid wheat (*Triticum turgidum* L.). In addition, a few wheat cultivars have other vernalization genes such as *RN-B4* and *VRN-D5* that are not linked to the *VRN-1* genes (Goncharov, 2003; Kato *et al*., 2003) but are also dominant for spring growth habit. *VRN-B4* is located on the short arm of wheat chromosome 7B. Yan *et al*. (2006) named it *VRN-B3*; it is orthologous to the barley vernalization gene *VRN-H3*.

A review of literature shows that no study has been performed to analyze allelic variation for vernalization requirement genes in Iranian wheat landraces. In view of the lack of information on *Vrn* alleles in Iranian wheat landraces, we examined the *Vrn-B1, Vrn-D1, Vrn-B3* genotypes of 395 wheat landraces collected from various regions of Iran. An increased understanding of the allelic variation of *Vrn* genes in Iranian wheat landraces will be useful for more effective development of wheat cultivars adapted to various environments.

MATERIALS AND METHODS

Plant materials

A total of 395 Iranian wheat landraces, including 154 spring, 193 winter, 46 of unknown growth habit, and 2 facultative genotypes (based on information from a CIMMYT greenhouse experiment), plus 2 standard cultivars, Chinese Spring and Thatcher, were used. Seeds of the plant materials were provided by CIMMYT's Wheat Collection.

Molecular analysis

Leaf tissues from 10 greenhouse grown seedlings per genotype were pooled and genomic DNA was extracted according to the CTAB method (Saghai-Maroof *et al*., 1984). PCR primers reported by Yan *et al*. (2004a), Fu *et al*. (2005), and Yan *et al*. (2006) were used to examine the promoter region for the presence of dominant or recessive alleles in *VRN-B1*, *VRN-D1*, and *VRN-B3* loci (Table 1).

PCR reactions were performed in a 10-µl volume in a BioRad thermocycler containing 0.6 µl of each of the 5 µmol/l forward and reverse primers, 4 µl of

Table 1. Primer sequences, annealing temperatures, and expected PCR product sizes for detecting alleles at the *VRN* loci in wheat.

Marker	Primer	Sequence	Expected size (bp)	Annealing TM	PCR profile*
VRN-B1	VRN1BF	5'-CAGTACCCCTGCTACCAGTG-3'	1000	58	Touch
promoter region	VRN1-R	5'-TGCACCTTCCC(C/G)CGCCCCAT-3'			down
VRN-D1	VRN1DF	5'-CGACCCGGGCGGCACGAGTG-3'	750	60	Touch
promoter region	VRN1-R	5'-TGCACCTTCCC(C/G)CGCCCCAT-3'			down
TDB	Intr1/B/F	5'-CAAGTGGAACGGTTAGGACA-3'	709	58	Ramp
VRN-B1 deletion	Intr1/B/R3	5'-CTCATGCCAAAAATTGAAGATGA-3'			
TDE	Intr1/D/F	5'-GTTGTCTGCCTCATCAAATCC-3'	1671	61	Ramp
VRN-D1 deletion	Intr1/D/R3	5'-GGTCACTGGTGGTCTGTGGC-3'			
VRN-D1	Intr1/D/F	5'-GTTGTCTGCCTCATCAAATCC-3'	997	61	Ramp
non-deletion	Intr1/D/R4	5'-AAATGAAAAGGAACGAGAGCG-3'			
VRN3	VRN4-B-INS-F	5'-CATAATGCCAAGCCGGTGAGTAC-3'	1200	57	Ramp
marker	VRN4-B-INS-R	5'-ATGTCTGCCAATTAGCTAGC-3'			
	VRN4-B-NOINS-F	5'-ATGCTTTCGCTTGCCATCC-3'	1140	57	Ramp
	VRN4-B-NOINS-R	5'-CTATCCCTACCGGCCATTAG-3'			

***Ramp: 1, 94℃, 5 min; 2, 94℃, 30 s; 3, 0.5℃/s to annealing TM; 4, annealing TM 30 s; 5, 0.2℃/s to 72℃; 6, 2℃, 30s; 7, go to step 2, 39 more times; 8, 72℃, 5 min; 9, 4℃, 5 min.**

Touch down: 1, 95℃, 5 min; 2, 96℃, 1 min; 3, 68℃, 5 min, -2.0℃/cycle; 4, 72℃, 1 min; 5, go to step 2, 4 more times; 6, 96℃, 1 min; 7, 58℃, 2 min, -2.0℃/cycle; 8, 72℃, 1 min; 9, go to step 6, 4 more times; 10, 96℃, 1 min; 11, 50℃, 1 min; 12, 72℃, 1 min; 13, go to step 10, 24 more times; 14, 72℃, 5 min; 15, 4℃, 5 min.

PCR ready MasterMix (Amplicon), 2.8 µl of template DNA, and 3 µl of sterile water. The PCR programs for each primer pair are presented in Table 1. The amplification products were separated by electrophoresis on 2% agarose gel at 100 V, stained with ethidium bromide, and subsequently visualized using 5 UV lights. A size marker of 50/100 bp plus (Fermentas) was used to estimate the size of different alleles amplified at *VRN* loci. Amplification experiments were repeated to confirm the allelic composition result.

RESULTS AND DISCUSSION

VRN-B1 promoter region marker

Allelic variation at the promoter region of *VRN-B1* gene in 395 Iranian wheat landraces was tested using primers VRN1BF and VRN1R. Amplification of genomic DNA from the promoter region of the landraces using these primers showed the presence of PCR products 980 bp and 1050 bp in length (Figure 1A). A total of 133 genotypes including 49 winter, 80 spring, 3 of unknown growth habit, and 1 facultative genotype, as well as cultivars Chinese

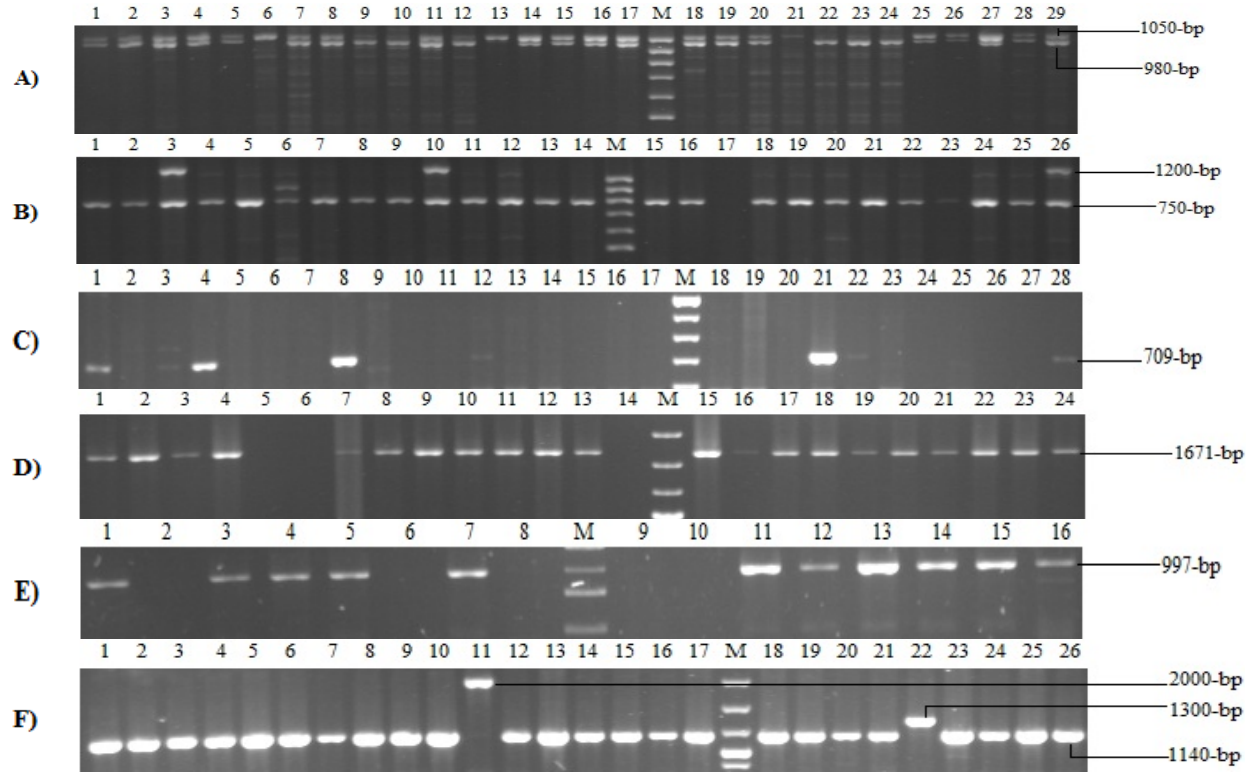

Fig. 1. Banding pattern of vernalization genes in Iranian wheat landraces. A) *Vrn-B1* promoter region (VRN1BF and VRN1R primers). B) *Vrn-D1* promoter region (VRN1DF and VRN1R primers). C) *Vrn-B1* locus (Intr1/B/F and Intr1/B/R3 primers). D) *Vrn-D1* locus (Intr1/D/F and Intr1/D/R3 primers). E) *vrn-D1* locus (Intr1/D/F and Intr1/D/R4 primers). F) *vrn-B3* locus (VRN4-B-NOINS-F and VRN4-B-NOINS-R). M: GeneRuler 100-bp plus DNA ladder marker (Fermentas).

Spring and Thatcher, were heterozygous for 980-bp and 1050-bp fragments. In 13 spring, 16 winter, 4 unknown, and 1 facultative genotype, only the 1050-bp fragment was observed; however, the 980-bp fragment was amplified in 126 landraces, including 40 spring, 68 winter, and 18 genotypes of unknown growth habit.

Yan *et al*. (2004a) used specific forward primers for the A, B, and D genomes; VRN1AF, VRN1BF, and VRN1DF in combination with the same degenerated reverse primer VRN1R, located within the first 20 bp of *VRN-1* exon 1 to characterize allelic variation at the *VRN-1* promoter region in polyploid wheat. This degenerated primer was used to accommodate a polymorphism between *VRN-1* genes in the A, B and D genomes. No sequence differences were found between the dominant *Vrn-B1* allele from TDB (isogenic lines with spring growth habit determined by dominant *Vrn-B1*) and the recessive *vrn-B1* allele from TDC (winter growth habit and recessive alleles at the three *VRN-1* genes) in the first 591-bp upstream from the start codon.

Analysis of partial sequences of the *VRN-B1* promoter region (400-bp including the CArG box and the putative TATA-box region) in four hexaploid varieties known to have the *Vrn-B1* dominant allele also showed no differences with the *VRN-B1* promoter sequences from TDB and TDC.

The absence of differences in the promoter sequences between the dominant *Vrn-B1* allele and its correspondent recessive alleles supports the hypothesis that additional regulatory sites are present in this gene outside the promoter region which was amplified by this pair of primers. However, we were able amplify two different fragments from the promoter region of *VRN-B1* gene in Iranian wheat landraces. Sequence analysis of these fragments may result in a new hypothesis and new *VRN-B1* alleles.

***VRN-D1* promoter region marker**

Specific D genome primer VRN1DF along with degenerated primer VRN1R (Yan *et al*., 2004a) were used to amplify the promoter region of *VRN-1* gene from the D genome of Iranian wheat landraces. Two fragments 750 bp and 1200 bp in length were amplified in 9 spring and 12 winter genotypes, plus 2 of unknown growth habit (Figure 1B). Three hundred and thirty-four landraces including 113 spring, 176 winter and 45 of unknown growth habit, as well as cvs. Chinese Spring and Thatcher, were homozygous for the 750-bp fragment. In five landraces (two winter, two facultative, and one spring), only the 1200-bp fragment was amplified, indicating the possibility of insertion in the promoter region of *VRN-1* gene in D genome of these landraces.

Similarly, Yan *et al*. (2004a), using VRN1DF and VRN1R primers, did not find any sequence differences in the promoter region of the dominant *Vrn-D1* allele from TDE and the recessive *vrn-D1* allele from TDC in the first 772-bp upstream from the start codon. They only reported one base difference (position -707 from the start codon) between the sequences obtained from the *VRN-D1* promoter region of hexaploid wheat and the D genome of *T. tauchii*. Golovnina *et al*. (2010), in characterizing *VRN1* loci in wild and cultivated wheat, reported that *VRN-1* promoter fragments of winter cultivars and the B and D genomes of spring wheat Mironovskaya Yarovaya contained a common sequence for certain genomes variability that was observed in recessive alleles of winter wheat near-isogenic line Triple Dirk C.

TDB *VRN-B1* deletion marker

The B genome-specific pair of primers Intr1/B/F and Intr1/B/R3 was used to test for the presence of the large deletion in the dominant *Vrn-B1* allele in Iranian wheat landraces (Figure 1C). Using these primers, a 709-bp fragment was found in 63 spring and 69 winter genotypes, plus 19 of unknown growth habit and 1 facultative genotype, suggesting that these accessions all have the same deletion. The presence of this allele in winter wheat may be due to growth habit misclassification in this set of Iranian wheat landraces. It could be argued that the *Vrn-B1a* allele was amplified in most landraces because *Vrn-B1* is the least effective allele and *Vrn-B1a* allele might be facultative to winter wheat. The lack of amplification in the remaining landraces may suggest that they carry the recessive vrn-B1 allele.

Fu *et al*. (2005), in analyzing *VRN-1* allele combinations in wheat varieties grown in Argentina and California, detected deletions in the first intron of the *VRN-B1* gene in 20 varieties. However, they did not observe *Vrn-B1* deletion in 24 accessions of tetraploid varieties grown in Argentina and California. The frequency of the *Vrn-B1* allele in varieties from Argentina and California was 66.1 and 49.1%, respectively. Iqbal *et al*. (2007), in a study of 40 spring wheat cultivars from Canada, confirmed the presence of dominant allele *Vrn-B1* in 20 genotypes. Zhang *et al*. (2008) identified dominant *Vrn-B1* allele in 73 of 273 Chinese wheat cultivars. Nowak and Kowalczyk (2010), in a study of 43 common wheats from Polish registered germplasm, were able to amplify dominant allele *Vrn-B1* in 13 winter and 11 spring cultivars.

TDE *VRN-D1* deletion marker

The D genome-specific pair of primers Intr1/D/F and Intr1/D/R3 amplified a 1671-bp band, which is an expected band size only when the dominant allele *Vrn-D1* with the large intron deletion is present (Figure 1D). This fragment was observed in 266 genotypes, including 116 spring, 120 winter, 28 of unknown growth habit, 2 facultative genotypes and cv. Chinese Spring.

The Intr1/D/F and Intr1/D/R3 pair of primers has been used in various studies to identify dominant allele *Vrn-D1* in wheat collections. Iqbal *et al*. (2007) did not observe dominant allele *Vrn-D1* in 40 Canadian spring wheat cultivars, and neither did Nowak and Kowalczyk (2010) in Polish common wheat cultivars. However, Zhang *et al*. (2008) reported the presence of dominant allele *Vrn-D1* in 105 of 278 Chinese wheat cultivars, and Iqbal *et al*. (2011) also reported a 1650-bp fragment in 35 of 59 spring bread wheat cultivars from Pakistan.

***VRN-D1* non-deletion marker**

The pair of primers Intr1/D/F and Intr1/D/R4 was used to amplify sequences with no deletion of the large fragment in the 1st intron of *VRN-D1* locus in Iranian wheat landraces. Using this pair of primers, a 997-bp fragment characteristic of the recessive *vrn-D1* allele was amplified in 127 genotypes consisting of 46 spring, 68 winter, 12 of unknown growth habit, 1 facultative genotype, and cv. Thatcher (Figure 1E), demonstrating that they carry the winter growth habit allele in this locus. Zhang *et al*. (2008)

reported the recessive allele *vrn-D1* in 173 of 278 Chinese wheat cultivars, and Iqbal *et al*. (2011) observed this allele in 24 of 59 spring wheats from Pakistan.

***VRN-B3* markers**

VRN-B3, a dominant allele for spring growth habit located on chromosome arm 7BS, was identified in the cultivar Hope. The dominant allele *Vrn-B3* has a retrotransposon insertion in the promoter region of a gene similar to *Arabidopsis FT* (Yan *et al*., 2006). The dominant allele *Vrn-B3*, defined by amplifying a 1200-bp fragment with primers VRN4-B-INS-F and VRN4-B-INS-R, was absent from all studied genotypes.

The absence of dominant allele *Vrn-B3* was further confirmed using a pair of primers VRN4-B-NOINS-F and VRN4-B-NOINS-R that amplified a 1140-bp fragment characteristic of the recessive allele *vrn-B3* in 366 genotypes including 145 spring, 178 winter, 43 of unknown growth habit, and cvs. Chinese Spring and Thatcher. In addition, two novel 1300-bp and 2000-bp fragments were amplified using this pair of primers (Figure 1F). The 1300-bp band was observed in four winter, four spring, and one genotype of unknown growth habit. Three spring and two winter genotypes showed the 2000-bp fragment. Spring landrace Ghoochan6 was heterozygous for 1300-bp and 2000-bp fragments, and winter landrace Sabzevar8 was heterozygous for 1140-bp and 1300-bp alleles. Amplification of new alleles in Iranian landraces may be due to new retrotransposon insertions in the promoter region of the *VRN-B3* gene which have not been reported.

Zhang *et al*. (2008), when analyzing the allelic variation of *VRN-B3* gene in 278 Chinese wheat cultivars, reported the dominant allele *Vrn-B3* (a 1200-bp fragment) only in cultivars Longfumai1 and Liaochun10, whereas all other cultivars showed an 1140-bp amplification fragment which is characteristic of the recessive *vrn-B3*. Similarly, the *Vrn-B3* allele was not found in Pakistani spring wheat cultivars, but all of the cultivars amplified an 1140-bp fragment characteristic of the *vrn-B3* allele (Iqbal *et al*., 2011).

Allelic variation at the *VRN-B1*, *VRN-D1*, and *VRN–B3* loci in Iranian wheat landraces

Among the *VRN* alleles detected in Iranian wheat landraces, recessive *vrn-B3* had the highest frequency (92.66%), followed by spring habit *Vrn-D1* (67.35%), dominant *Vrn-B1* (38.48%), and winter habit *vrn-D1* (32.15%) (Figure 2). The frequency of recessive *vrn-B3* in spring and winter genotypes was (94.16%) and (92.23%), followed by dominant *Vrn-D1* (75.33%) and (62.18%) (Figure 3). Iqbal *et al*. (2007) reported that the *Vrn-B1* allele was found in (50%) of Canadian spring wheat cultivars, whereas the *Vrn-D1* allele was absent in all these cultivars. Iqbal *et al*. (2011) reported *Vrn-B1* was the most frequent allele (64%), followed by *Vrn-D1* (61%), among the spring habit alleles in Pakistani wheat cultivars.

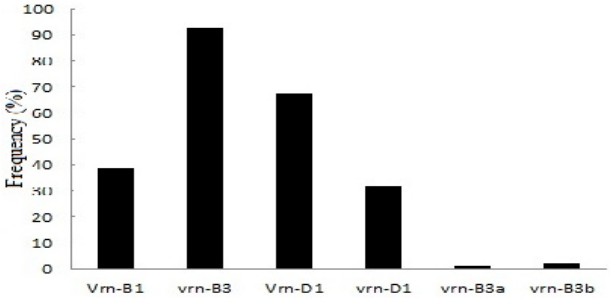

Fig. 2. Frequency of *VRN-B1*, *VRN-B3*, and *VRN-D1* loci in all the Iranian wheat landraces.

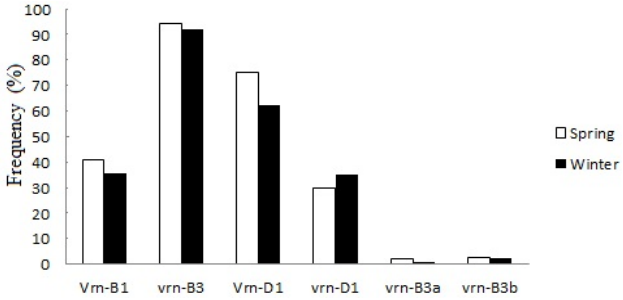

Fig. 3. Frequency of *VRN-B1*, *VRN-B3*, and *VRN-D1* loci in spring and winter genotypes.

Allelic combinations of the *VRN* loci in 395 Iranian wheat landraces are presented in Tables 2 and 3. Fifty-four accessions consisting of 31 spring and 19 winter genotypes carried combinations of *Vrn-B1, Vrn-D1, vrn-B3* alleles. A combination of alleles *Vrn-B1*, *vrn-D1*, *vrn-B3* was observed in 33 spring and 41 winter accessions. The dominant allele *Vrn-B3* was not amplified in any Iranian wheat landraces. In addition, seven out of ten accessions with novel alleles in the *vrn-B3* gene are from western (4) and southeastern (3) Iran (Table 3). Moreover, it was very surprising to observe both dominant *Vrn-D1* (with the intron-1 deletion) and recessive *vrn-D1* (without any deletion) in 29 accessions, including 11 spring, 15 winter, 2 unknown, and 1 facultative (Tables 2 and 3).

These findings indicate that the *Vrn-B1* and *Vrn-D1* alleles can play an important role in determining the growth habit and adaptation of CIMMYT germplasm. Van Beem *et al*. (2005) analyzed genetic factors influencing the developmental rate of globally important CIMMYT wheat cultivars and found that wheat materials developed at CIMMYT have a high frequency of spring habit allele *Vrn-D1* and are adapted to tropical and sub-tropical regions

Table 2. Distribution of *Vrn-B1*, *Vrn-D1*, and *Vrn-B3* alleles in wheat landraces with different growth habit.

Allelic combination	Total	Growth habit			
		Spring	Winter	Facultative	Unknown
Vrn-D1 vrn-D1	29	11	15	1	2
Vrn-B1 Vrn-D1	57	32	20	1	4
Vrn-B1 vrn-D1	93	36	47	1	9
Vrn-B1 Vrn-D1 vrn-B3	54	31	19	0	4
Vrn-B1 vrn-D1 vrn-B3	83	33	41	0	9
Vrn-B1 Vrn-D1 vrn-B3b	2	1	1	0	0
Vrn-B1 vrn-D1 vrn-B3b	6	2	4	0	0
Vrn-B1 Vrn-D1 vrn-B3a	1	0	1	0	0
Vrn-B1 vrn-D1 vrn-B3a	1	1	0	0	0

Table 3. Allelic variation at *VRN-B1*, *VRN-D1*, and *VRN-B3* loci in Iranian wheat landraces.

Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*	Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*
Iran1	D	-	R	Birjand1-w	-	D	R
Urmia1-w	D	D	R	Bojnourd2-w	-	D	R
Iran2	-	D	R	Torbat-Heidarieh1-w	-	D	R
Iran3	-	D	R	Bojnourd3-s	-	D	-
Iran4	D	R	R	Feridan1-s	D	R	R
Malayer1-w	-	D	R	Borujen1-w	-	D	R
Arak1-w	D	D,R	R	Yazd1-w	-	-	R
Iran5	-	D	R	Yazd2-w	-	D	R
Iran6	D	-	R	Shahre-Kord1-w	-	D	R
Sanandaj1-s	D	R	R	Shahreza1-w	-	D	R
Dareh-Gaz1-w	-	R	R	Shahreza2-w	-	D	R
Kermanshah1-s	-	D	-	Shirvan1-w	D	R	R
Gazvin1-s	D	R	R	Iran8	D	D	R
Shah-Abad1-s	D	D,R	R	Shahreza3-w	D	D	R
Kerend1-s	D	D,R	-	Borujen3-w	-	D	R
Saveh1-s	-	D	-	Borujen4-w	-	D	R
Gazvin2-s	D	R	R	Semirom1-s	-	D	R
Gazvin3-w	D	R	R	Ghoochan2-s	D	R	R
Gilane-Gharb1-w	D	R	Rb	Birjand3-s	-	D	R
Gilane-Gharb2-w	D	R	R	Yazd3-w	-	D	R
Ilam1-w	D	R	R	Yazd4-w	D	D	R
Ilam2-w	D	-	R	Shahreza4-w	D	D	Ra
Malayer2-w	-	R	R	Birjand4-w	-	D	R
Hamedan1-s	-	D	R	Varamin1-w	-	D	R
Gorgan1-s	-	-	-	Semirom2-w	-	D	R
Kashmar1-w	-	-	R	Shahreza5-w	D	D	R
Kashmar2-w	-	D	R	Shahreza6-w	D	-	R
Sabzevar1-w	-	-	R	Shiraz1-w	-	D	R
Sabzevar2-w	D	D	R	Shiraz2-s	D	D	R
Ardakan1-w	-	D	R	Shiraz3-s	D	D,R	R
Iran7	D	-	-	Iran9	D	R	R
Sabzevar3-w	-	R	R	Fasa1-s	-	D	R
Torbat-Jam1-w	-	D	R	Neiriz1-w	-	D	R
Ghoochan1-w	-	D	R	Shiraz4-w	-	D	R
Esfahan1-w	-	D	R	Shiraz5-s	D	R	R
Ardakan2-w	-	D	R	Hasht-Rood1-w	D	R	R
Neishabour1-w	-	D	R	Kerman1-w	D	D	R
Neishabour2-s	D	R	R	Ardabil1-s	D	R	R
Dastjerd1-s	-	D	R	Urmia2-f	D	D,R	-
Esfahan2-w	-	D	R	Urmia3-w	D	R	Rb
Bojnourd1-w	D	D,R	R	Urmia4-w	D	D	R

D and R indicate and dominant recessive alleles at the *VRN-B1*, *VRN-D1*, and *VRN-B3* loci, respectively. Ra: new allele with 2000-bp, Rb: new allele with 1300-bp in *vrn-B3* gene. S: spring, w: winter, f: facultative growth habit, - (dash) : no amplification.

Table 3. (continued)

Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*	Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*
Kerman2-w	-	D	R	Torbat-Jam5-w	-	D,R	R
Sirjan1-w	-	D	R	Naghadeh1-s	D	R	R
Kerman3-w	-	D	R	Iran12	D	R	R
Kerman4-w	D	D	R	Esfahan8-w	-	R	R
Shahreza7-w	-	D	R	Esfahan9-w	-	D	R
Shiraz6-w	-	R	R	Borujerd1-w	-	-	R
Moghan (Gearmi)1-w	-	D	R	Borujerd2-s	D	D	R
Urmia5-w	-	D	R	Urmia8-w	D	R	Rb
Ardabil2-w	D	R	R	Mahabad1-s	D	D	R
Tabriz1-w	D	R	-	Mahabad2-s	-	D	R
Mianeh1-w	-	D	R	Ghoochan5-s	-	D	R
Bandar-Abbas1-w	-	D	Ra	Ghoochan6-s	D	R	Ra,Rb
Shiraz7-s	-	D	R	Mashhad4-s	-	R	R
Lenjan1-w	-	D	R	Mashhad5-w	D	R	R
Esfahan3-w	-	D	R	Fooman1-s	D	R	R
Urmia6-w	-	D	R	Birjand5-w	-	D	R
Urmia7-w	-	D	R	Birjand6-w	-	-	R
Ghoochan3-f	-	D	-	Birjand7-w	-	-	R
Iran10	-	D	R	Feridan2-w	D	D	R
Lenjan2-w	-	D	R	Bojnourd4-s	-	D	R
Esfahan4-w	-	D	R	Bojnourd5-s	-	D	R
Esfahan5-w	D	R	R	Dareh-Gaz2-s	-	D	R
Esfahan6-w	-	D	R	Ghoochan7-s	D	D	R
Mashhad1-w	-	D	R	Sarakhs1-s	-	D	Ra,Rb
Ghoochan4-w	-	D	R	Shahrud1-s	-	D	R,Ra
Mashhad2-s	-	D	R	Tabas1-w	-	D	R
Najaf-Abad1-w	-	D	R	Meimeh1-w	-	D	R
Torbat-Jam2-s	-	D	R	Meimeh2-w	-	D	R
Torbat-Jam3-w	D	D,R	R	Ghoochan8-s	-	D	R
Torbat-Jam4-w	-	D	R	Esfahan10-w	-	D	R
Damghan1-w	D	D	R	Shahrud2-s	D	D,R	R
Shah-Abad2-w	D	-	R	Meimeh3-w	-	-	R
Sanandaj2-w	D	R	R	Esfahan11-w	-	-	R
Zanjan1-w	D	R	R	Shahrud3-s	-	D	R
Zanjan2-s	-	D	R	Semnan1-w	-	D	R
Mashhad3-s	D	-	R	Najaf-Abad2-s	-	D	R
Esfahan7-w	-	-	R	Najaf-Abad3-w	-	D	R
Sanandaj3-s	-	D,R	R	Shah-Abad3-s	-	D	R
Iran11	-	D	R	Mashhad6-s	D	R	R
Khonsar1-w	-	D,R	R	Saghez1-w	D	-	R
Damghan2-w	D	R	R	Gazvin4-w	D	-	R
Shah-Abad4-s	-	D	R	Toyserkan1-w	D	R	R
Gazvin5-w	D	R	R	Toyserkan2-s	D	D,R	R
Gazvin6-s	D	D,R	R	Torbat-Heidari2-s	D	D	R
Gazvin7-w	D	R	R	Hamedan3-w	-	D,R	R
Saghez2-w	D	-	R	Iran14	D	-	R
Shah-Abad5-w	D	R	R	Sabzevar5-w	D	-	R
Sabzevar4-s	D	D	R	Iran15	D	-	R
Ghoochan9-s	-	D	R	Sabzevar6-s	-	D	R
Torbat-Jam6-s	D	D	R	Sabzevar7-s	-	D,R	R
Birjand8-w	-	D	R	Iran16	D	-	R

D and R indicate and dominant recessive alleles at the *VRN-B1*, *VRN-D1*, and *VRN-B3* loci, respectively. Ra: new allele with 2000-bp, Rb: new allele with 1300-bp in *vrn-B3* gene. S: spring, w: winter, f: facultative growth habit, - (dash) : no amplification.

Table 3. (continued)

Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*	Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*
Birjand9-s	D	D	R	Sabzevar8-w	D	D,R	R,Rb
Semirom3-w	D	D,R	R	Iran17	D	-	Rb
Ardestan1-w	D	R	R	Sabzevar9-s	-	D	R
Rafsanjan1-w	-	D	R	Bojnourd6-s	-	D	R
Torbat-Jam7-w	-	D	R	Iran18	-	D	R
Neishabour3-w	D	R	R	Iran19	-	D	R
Shirvan2-w	D	R	R	Sabzevar10-w	D	R	R
Iran13	D	R	R	Kashmar3-s	D	D	R
Arak2-s	-	D	R	Yazd5-s	D	D	R
Ghasre-Shirin1-w	-	D	R	Iran20	D	R	R
Ghasre-Shirin2-w	-	D	R	Yazd6-w	D	R	R
Gilane-Gharb3-w	-	D	R	Sabzevar11-w	D	R	R
Gilane-Gharb4-s	-	D	R	Iran21	D	R	R
Gazvin8-s	-	D	R	Iran22	-	D	R
Mahidasht1-w	-	D	R	Sabzevar12-w	-	D	R
Gorgan2-s	-	D	R	Sabzevar13-s	D	R	R
Kermanshah2-w	-	D	R	Feridan3-w	D	R	R
Sanandaj4-s	-	D	R	Sabzevar14-s	D	D	R
Shah-Abad-Gharb1-w	-	D	R	Iran23	-	D	-
Saveh2-w	-	D	R	Ardakan3-s	D	D	R
Hamedan2-w	-	D	R	Iran24	D	R	R
Sanandaj5-s	D	D	R,Rb	Mashhad7-s	D	D	R
Mahidasht2-s	-	D	R	Najaf-Abad4-w	D	R	R
Kermanshah3-w	D	D	R	Iran25	-	-	R
Sanandaj6-s	-	D	R	Iran26	D	D	R
Maragheh1-w	-	D	R	Iran27	D	D,R	R
Kermanshah4-w	D	D	R	Ghoochan10-w	D	R	R
Sanjabi1-w	-	D	R	Esfahan12-w	-	D	R
Divan-Dareh1-w	-	D	R	Iran28	-	D	R
Malayer3-s	-	D,R	R	Iran29	D	R	R
Nahavand1-w	D	-	R	Ardakan4-w	-	R	R
Mashhad8-w	-	D	R	Astara1-w	D	R	R
Mashhad9-w	-	D	R	Shahi1-w	-	R	R
Mashhad10-s	D	R	R	Esfahan14-w	D	R	R
Sabzevar15-s	-	D	R	Torbat-Jam8-s	D	D,R	R
Sabzevar16-w	-	D	R	Fariman1-w	-	-	R
Mashhad11-w	-	D	R	Gonabad1-w	-	D	R
Iran30	-	D	R	Gorgan3-s	-	D	R
Mashhad12-w	-	D	R	Semnan2-s	-	D	R
Ghoochan11-w	-	D	R	Shah-Abad6-w	D	R	R
Iran31	-	D	R	Mashhad13-s	D	D	R
Iran32	-	D	R	Gazvin9-w	D	R	R
Neishabour4-w	-	D	R	Sabzevar17-w	-	D	-
Bojnourd7-w	-	D	R	Ardakan5-w	-	D,R	-
Iran33	-	D	R	Bojnourd11-w	D	R	-
Shahre-Kord3-w	-	D	R	Shahre-Kord5-w	D	D,R	R
Neishabour5-w	-	D	R	Torbat-Heidarieh4-w	-	D	R
Neishabour6-w	-	D,R	R	Naein1-w	-	D	R
Bojnourd8-s	D	D	R	Shahre-Kord6-w	D	D,R	R
Bojnourd9-w	D	-	R	Semirom4-w	D	R	R
Bojnourd10-s	-	D	R	Shirvan3-s	D	D	R

D and R indicate and dominant recessive alleles at the *VRN-B1*, *VRN-D1*, and *VRN-B3* loci, respectively. Ra: new allele with 2000-bp, Rb: new allele with 1300-bp in *vrn-B3* gene. S: spring, w: winter, f: facultative growth habit, - (dash) : no amplification.

Table 3. (continued)

Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*	Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*
Neishabour7-w	-	D,R	R	Dareh-Gaz3-s	-	D	R
Iran34	-	D	R	Ghoochan12-s	-	D	R
Hamedan4-s	-	D	R	Ghasre-Shirin3-s	-	D	R
Iran35	-	D	R	Malayer4-s	-	D	R
Iran36	-	D	R	Mahi-Dasht3-s	-	D	R
Iran37	-	D	R	Kermanshah5-w	-	D	R
Iran38	-	D	R	Gazvin10-s	D	D	R
Tabas2	-	D	R	Varamin2-s	D	R	R
Iran39	D	D	R	Iran40	-	D,R	R
Shahre-Kord4-s	-	R	R	Gilane-Gharb5-s	D	D	R
Niriz2-w	-	D	R	Hamedan6-s	D	R	R
Shah-Roud4-w	-	D	R	Esfahan15-s	D	D	R
Hasht-Rood2-s	D	R	R	Sanjabi2-w	D	R	-
Arak3-s	D	R	R	Neishabour8-s	-	D	R
Sanandaj7-w	D	R	R	Birjand10-w	-	D	-
Hamedan5-s	D	D	R	Ghasre-Shirin4-s	-	D	R
Tabas3-s	-	D	R	Shah-Abad7-s	D	D	R
Esfahan13-w	-	D	R	Bojnourd12-w	-	D	R
Borujen5-w	D	R	R	Kashmar4-s	-	D	R
Torbat-Heidar3-w	-	R	R	Kashmar5-w	D	R	R
Borujen6-w	-	R	R	Sabzevar18-s	-	D	R
Yazd7-s	-	D	R	Iran51-s	D	R	R
Ghoochan13-s	D	R	R	Iran52-s	D	R	R
Tabas4-s	-	D	R	Zanjan3-s	-	R	R
Iran41	-	D	R	Shahrood5-s	-	D	R
Hamedan7-w	-	D	R	Semnan3-s	-	D	R
Tabas5-s	-	D	R	Kerman5-s	-	D	R
Esfahan16-s	D	R	R	Zahedan1-s	-	D	R
Saghez3-s	D	R	R	Zahedan2-s	-	D	R
Fariman2-w	-	D,R	R	Zahedan3-s	-	D	R
Iran42	-	D	R	Zahedan4-s	-	D	R
Bojnourd13-w	-	D	R	Esfahan18-s	-	D	R
Sabzevar19-s	-	D	R	Esfahan19-s	-	D	R
Iran43	-	D	R	Esfahan20-s	-	D	R
Neiriz4-w	D	R	R	Esfahan21-s	-	D	R
Shiraz8-s	D	R	R	Esfahan22-s	D	D	R
Shiraz9-s	D	R	R	Shahre-Kord7-s	-	D	R
Maragheh2-s	D	D	R	Mashhad14-s	D	-	-
Iran44	-	D	R	Shahre-Kord8-s	-	D	R
Urmia9-w	-	D	R	Mashhad15-s	-	D	R
Babol1-w	D	R	R	Mashhad16-s	-	D	R
Esfahan17-w	-	R	R	Mashhad17-s	-	R	R
Damghan3-w	-	D,R	R	Mashhad18-s	-	D,R	R
Iran45	-	R	R	Mashhad19-s	-	D	R
Gazvin12-w	-	R	-	Kerman7-s	-	R	R
Iran46-s	-	R	R	Kerman8-s	-	D	R
Iran47-s	D	R	Rb	Kerman9-s	-	D	R
Hamedan8-w	-	R	-	Kerman10-s	-	D	R
Iran48	-	R	R	Kerman11-s	-	D	R
Gazvin13-w	-	R	-	Esfahan23-s	-	D	R
Iran49-s	D	R	R	Esfahan24-s	D	D	R

D and R indicate and dominant recessive alleles at the *VRN-B1*, *VRN-D1*, and *VRN-B3* loci, respectively. Ra: new allele with 2000-bp, Rb: new allele with 1300-bp in *vrn-B3* gene. S: spring, w: winter, f: facultative growth habit, - (dash) : no amplification.

Table 3. (continued)

Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*	Genotype	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*
Iran50-s	**D**	**R**	**R**	**Yazd8-s**	**-**	**D**	**R**
Hamadan9-w	**-**	**R**	**-**	**Tehran2-s**	**-**	**D**	**R**
Tehran1-s	**-**	**D**	**R**	**Chinese spring**	**-**	**D**	**R**
Birjand11-s	**-**	**D**	**R**	**Thatcher**	**-**	**R**	**R**
Sarakhs2-s	**D**	**R**	**R**				

D and R indicate and dominant recessive alleles at the *VRN-B1*, *VRN-D1*, and *VRN-B3* loci, respectively. Ra: new allele with 2000-bp, Rb: new allele with 1300-bp in *vrn-B3* gene. S: spring, w: winter, f: facultative growth habit, - (dash) : no amplification.

of the world. Iwaki *et al*. (2001), in a study of 272 wheat cultivars from different geographical regions, showed that differences in *Vrn* genotypes are linked to their origin. They found that dominant allele *Vrn-A1* is the most frequent allele in European common wheat cultivars, spring habit allele *Vrn-B1* is moderately frequent, and dominant allele *Vrn-D1* is very rare.

In a study of 551 Chinese bread wheat cultivars, Sun *et al*. (2009) reported that the growth habit of 225 accessions was not completely predicted by their allelic status at the *Vrn-1* genes, suggesting that there were novel alleles or genes related to vernalization in wheat. Some as yet unknown genes affecting wheat's vernalization requirement have been assigned to locations on the chromosomes of homoeologous groups 1, 3 and 6 (Miura and Worland, 1994; Islam-Faridi *et al*., 1996; Law *et al*., 1998). In addition, the genetic background interacts with *Vrn-1* to affect growth habit (Danyluk *et al*., 2003).

Results of the present study indicate the need for conducting precise field and greenhouse evaluations for identifying growth habit, and also support the theory of the existence of other regulatory regions that determine the vernalization requirement in wheat.

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