Biological Forum – An International Journal **7**(2): 395-400(2015)

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Variation in the first intron of *VRN-1* gene in winter and spring Iranian wheat landraces

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(Received 29 May, 2015, Accepted 30 July, 2015)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Vernalization requirement in hexaploid wheat is largely controlled by a series of homoeologous *Vernalization (Vrn)* genes; *Vrn-A1, Vrn-B1,* and *Vrn-D1*. In this study, the first intron of *Vrn1* in 395 spring and winter Iranian wheat landraces were analyzed. A primer pair of Intr1/A/F2 and Intr1/A/R3 was used to survey the large deletion in the 1st intron of *Vrn-A1* locus and 710-bp and 1170-bp fragments were amplified in 14 and 21 genotypes, respectively. Both fragment were isolated and sequenced from spring and winter types and the sequences of 710-bp allele in winter and spring types showed 98% identity with four SNPs. Based on second primer pair, (Intr1/B/F and Intr1/B/R3), a fragment of 709-bp was amplified in 152 genotypes including 63 spring and 69 winter type. Sequence analysis revealed 7 SNPs between facultative and winter genotypes. A conservation region of 577-bp length with conservation coefficient of 1.00 was identified. In addition, a fragment of 1671-bp was amplified in 266 genotypes using Intr1/D/F and Intr1/D/R3 primer pair and a fragment of 997-bp based on Intr1/D/F-Intr1/D/R4 primer pair in 127 genotypes. In this fragment, a region of 435-bp with complete identity was detected. The analyzed sequences in Iranian wheat landraces showed high similarity with available sequences of known vernalization genes in NCBI databases.

Keywords: conserved region, growth habit, sequence identity, vernalization, wheat landraces

INTRODUCTION

Wheat is one of the most important staple food crops of the world, occupying 17% (one sixth) of crop acreage worldwide, feeding about 40% (nearly half) of the world population and providing 20% (one fifth) of total food calories and protein in human nutrition (Gupta *et al.*, 2008). Wheat landraces are potentially a useful germplasm resource of genetic variability for traits of interest such as stress resistance, tolerance, long term adaptation and grain quality characteristics (Strelchenko *et al.*, 2008). They are widely used by national, private and international breeding programs to derive genes for resistance to biotic and abiotic stresses and good quality (Rawashdeh *et al.*, 2010).

Many cereal crops, such as wheat, barley and oat, are divided into spring and winter types based on their growth habit. Winter varieties require an extended period of exposure to cold in order to flower, a process known as vernalization. In wheat, vernalization requirement is primarily controlled by alleles at three orthologous loci, *Vrn-A1, Vrn-B1* and *Vrn-D1*, which are located on the long arms of chromosomes 5A, 5B and 5D, respectively (Law *et al.*, 1975; Galiba *et al.*, 1995; Dubcovsky *et al.*, 1998). Dominant alleles of each locus are designated as *Vrn-A1, Vrn-B1*, and *Vrn-D1*, whereas the recessive alleles are designated as *Vrn-A1, vrn-B1,* and *Vrn-D1*. The presence of a dominant *Vrn-1* allele in any genome confers spring growth habit, whereas the presence of recessive alleles in the homozygous state across *Vrn-1* loci confers winter growth habit. (Santra *et al.*, 2009). Additional vernalization responsive genes, the *Vrn-2* series located on chromosomes 4B, 4D and 5A, *Vrn-B3* (located on chromosome 7BS) and *Vrn-D5* (located on chromosome 5D) have been identified in wheat (Goncharov, 2003; Kato *et al.*, 2003; Yan *et al.*, 2006). These genes interact to bridge the link between responses to cold and day length to regulate plant development (Trevaskis *et al.*, 2007).

At *Vrn-A1*, three distinct mutations were reported to confer spring growth habit: (i) insertions within the promoter region; (ii) deletions within the promoter region; and (iii) large deletions in intron 1. The dominant alleles at this locus, which are associated with promoter insertions, deletions and the deletion in intron1, are designated as *Vrn-A1a*, *Vrn-A1b*, and *Vrn-A1c*, respectively (McIntosh *et al.*, 2007). Fu *et al.* (2005) developed DNA markers, specific for the *Vrn-A1c, vrn-A1, Vrn-B1, vrn-B1, Vrn-D1,* and *vrn-D1* alleles, based on the presence or absence of deletions in intron 1 in the dominant and recessive alleles, respectively. These markers were used to determine *Vrn-1* allelic compositions of 117 spring wheat genotypes from Argentina and California.

Large deletions within intron 1 of the *Vrn-B1* and *Vrn-D1* genes also resulted in spring growth habit. For the *VRN-B1* and *VRN-D1* loci, changes in promoter sequence were not observed; their allelic variation is determined only by deletion within the first intron sequence (Fu *et al.,* 2005).

In wheat and barley, the determination of the vernalization requirement involves an epistatic interaction between the genetic loci *VRN-1* and *VRN-2* (Tranquilli and Dubcovsky, 2000). The *VRN-1* gene is dominant for the spring growth habit and it is up-regulated by vernalization in winter lines, whereas the *VRN-2* gene is dominant for the winter growth habit and is down-regulated by vernalization (Yan *et al.*, 2004).The sequences between dominant and recessive alleles, and the known epistatic interactions between these two genes, a molecular model was proposed in which the product of *VRN-2* acts as a repressor of *VRN-1* (Yan *et al.*, 2003). According to this model, mutations in the *VRN-2* protein (Yan *et al.* 2004) result in an inactive repressor.

In this study, we examined the *Vrn-A1*, *Vrn-D1* and *Vrn-B3* sequences of 395 wheat landraces collected from various regions of Iran. These results will be useful for more effective development of wheat cultivars adapted to various environments.

MATERIALS AND METHODS

A. Plant materials

A set of 395 wheat landraces from different geographical regions of Iran including 154 spring, 193 winter, two facultative wheat and 46 unknown growth type along with two standard cultivars; Chinese Spring and Thatcher were kindly provided by International Center for the Improvement of Maize and Wheat (CIMMYT) were used.

B. DNA preparation and molecular analysis

DNA from fresh leaves of 10 plants of each genotype was extracted using CTAB (cetyltrimethyl ammonium bromide) method (Saghai-Maroof *et al.,* 1984). PCR reactions for molecular analyses were performed in a total volume of 10 containing 2µl of template DNA, 0.1µl dNTP (10mM), 0.5µl of each primer, 0.3µl $MgCl_2$ (50mM), 5. 2µl 1XPCR Buffer and 0.11 µl Taq DNA polymerase. PCR primers reported by Fu *et al.* (2005) were used to examine the intron1 region for the presence of dominant or recessive alleles in *VRNA1*, *VRNB1* and *VRN-D1* loci (Table 1). Program used for thermocycling was ramp consisted of 1) 94°C for 5 min; 2) 94° for 30 s; 3) decrease 0.5°C/s to annealing temperature; 4) annealing temperature for 30 s; 5) increase 0.2°C/s to 72°; 6) 72° for 30 s; 7) go to step 2, 39 more times; 8) 72° for 5 min and 9) 4° for 5 min. 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.

C. Sequencing of selected fragments

After selecting specified bands on agarose gel, PCR products were loaded on 6% polyacrylamide gel and visualized by Silver nitrate staining. The fragments were isolated from gel. To increase density of isolated fragments, PCR was repeated using respective primers. To determine the accuracy of isolation, the amplified fragments along with size marker were loaded on 1% agarose gel. The isolated fragments used for direct sequencing.

Nucleotide sequences were aligned by using the CLUSTALW package running under BioEdit Ver. 7.2.5 available from www.mbio.ncsu.edu_BioEdit (Hall, 1999). Reliable sequences were blasted in NCBI database using blastn. Conserved regions in the sequence of spring, winter and facultative types were identified using DnaSpver 5.10 software (Rozas *et al.,* 2003).

Table 1: PCR markers for the different *VRN-1* alleles in wheat.

PCR marker	Primers	Sequence (5′ to 3′)	Expected size (bp)	Annealing temperature (□C)*
Vrn-A1 deletion	Intr1/A/F2	AGCCTCCACGGTTTGAAAGTAA	1170	58.9
	Intr1/A/R3	AAGTAAGACAACACGAATGTGAGA		
Vrn-B1 deletion	Intr1/B/F	CAAGTGGAACGGTTAGGACA	709	58.0
	Intr1/B/R3	CTCATGCCAAAAATTGAAGATGA		
TDE *Vrn-D1* deletion	Intr1/D/F	GTTGTCTGCCTCATCAAATCC	1671	61.0
	Intr1/D/R3	GGTCACTGGTGGTCTGTGC		
Vrn-D1 Non-deletion	Intr1/D/F	GTTGTCTGCCTCATCAAATCC	997	61.0
	Intr1/D/R4	AAATGAAAAGGAACGAGAGCG		

* Initial denaturation was performed at 94°C for 5 min, followed by 38 cycles of 94°C for 30 s, annealing at the indicated temperature for 30 s, and extension at 72°C for 1 min/kb, with a final extension at 72°C for 10 min

RESULTS AND DISCUSSION

A. VRN-A1 deletion marker

The pair of primer Intr1/A/F2 and Intr1/A/R3 was used to survey the large deletion of the 1st intron of *VRN-A1* locus and amplify *VRN-A1c* allele in 395 Iranian wheat landraces. Using this pair of primer, 710 and 1170 bp fragments were amplified in the genotypes (Fig. 1a).

The allele of 1170 bp was only amplified in 21 genotypes consisted of 18 spring, two winter and one genotype with unknown growth type and 710 bp fragment was only amplified in 11 spring, two winter and one genotype with unknown growth type. The fragment of 710 bp fragment was isolated and sequenced from spring and winter genotypes. The sequences alignment showed 98% identity between two growth types and four SNPs were detected (A8C, G10C, T633G and T638C) between them. Fig. 2 shows part of the aligned sequences with the position of SNPs. The sequences were blasted in NCBI databases and showed 100% identity with *Triticum aestivum VRN-A1* gene sequences in Hereward, Malacca and Claire cultivars (JF965397.1, JF965396.1 and JF965395.1). There was a conserved region with 621-bp length and conservation coefficient of 1.00.

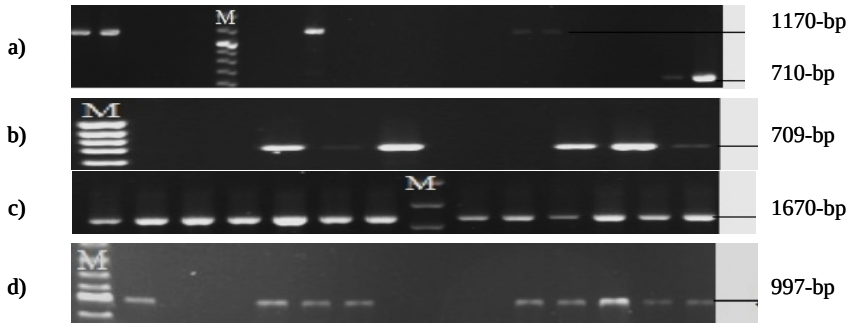

Fig. 1. Banding pattern of vernalization genes in Iranian wheat landraces. a) *Vrn-A1* locus (Intr1/A/F2 and Intr1/A/R3 primers). b) *Vrn-B1 locus* (Intr1/B/F and Intr1/B/R3 primers). c) *Vrn-D1* locus (Intr1/D/F and Intr1/D/R3 primers). d) *vrn-D1* locus (Intr1/D/F and Intr1/D/R4 primers).

S-Intr1AF2/Intr1AR3 TTCACACGGGCATTTATCTGCTTTGGCATCAAAGCTTTCATTTGAAAATT 547
W-Intr1AF2/Intr1AR3 TTCACACGGGCATTTATCTGCTTTGGCATCAAAGCTTTCATTTGAAAATT 550

S-Intr1AF2/Intr1AR3 TGCTACTACCACCTATATTTGTACTGACAATACCTTTGCATGGACCCACA 597
W-Intr1AF2/Intr1AR3 TGCTACTACCACCTATATTTGTACTGACAATACCTTTGCATGGACCCACA 600

S-Intr1AF2/Intr1AR3 CATTAGGTTTTAAAATGGTTCTCACATCGGGGTTTTTTTTTTAAAAAAAA 647
W-Intr1AF2/Intr1AR3 CATTAGGTTTTAAAATGGTTCTCACATCGGGGGTTTTCTTTTAAAAAAAA 650

Fig. 2. Part of sequence alignment for sequences of amplified fragments by pair of primers Intr1/A/F2 and Intr1/A/R3.

Sequencing of 1170 bp fragment from spring and winter landraces, cv. Chinese Spring and cv. Thatcher revealed several polymorphic features between sequences and average sequence identity was 40%. These sequences also showed identity with *VRN-A1* gene sequences of Hereward, Malacca and Claire cultivars, however the degree of identity was less than that of 710 bp fragment. Three conserved region were recognized in the sequences with 79, 77 and 82 bp length and average conservation coefficient of 0.32.

B. TDB VRN-B1 deletion marker

The B genome-specific primer pair Intr1/B/F and Intr1/B/R3 was used to test presence of the large deletion in dominant *Vrn-B1* allele in Iranian wheat landraces. A 709 bp fragment was amplified in 63 spring, 69 winter, 19 genotypes of unknown growth habit and one facultative landraces (Fig. 1b). Sequence analysis in spring, winter and facultative landraces revealed 98% between winter and facultative types with seven SNPs (T6C, T7C, C17A, C18A, C27A, A31G and C684G). Sequence identity between spring and winter types and spring and facultative genotype sequences was 85% and 86%, respectively. The differences between various growth habit (springs, winter and facultative) was in large gap in the first 40 nucleotides. Sequence comparison showed a conservation region of 577 bp length (Fig. 3) with conservation coefficient of 1.00.

Blast analysis revealed about 98% identity between these sequences and TriticumaestivumVRN-B1 gene sequences in Diamant2 cultivar, Triple Dirk B line, Lebsock and Triple Dirk C line (HQ130483.2, AY747603.1, FJ766015.1 and AY747604.1). There was also high identity between the isolated sequences and *VRN-B1* gene sequence of Triticum durum and *Triticum turgidum.*

Fig. 3. Conservation region in sequences of amplified fragment by pair of primers Intr1/B/F and Intr1/B/R3.

C. First intron markers of VRN-D1

A multiplex PCR with primer s of Intr1/D/F, Intr1/D/R3, and Intr1/D/R4 was used to investigate a region of intron-1 thought to span a cis-element implicated in the control of vernalization response. Primer pair of Intr1/D/F and Intr1/D/R3 amplified a 1671-bp fragment, which is an expected size for the dominant allele of *Vrn-D1* with the large intron deletion (Fig. 1c), in 266 genotypes including 116 spring, 120 winter, 28 of unknown growth habit, 2 facultative genotypes and cv. Chinese Spring. Primer pair Intr1/D/F-Intr1/D/R4 designed based on region of intron-1 amplified product of 997 bp in 127 genotypes consisting of 46 spring, 68 winter, 12 of unknown growth habit, one facultative genotype, and cv. Thatcher (Fig. 1d). The amplified was sequenced from spring, and facultative landraces and cv. Chinese Spring. The Sequences of facultative genotype and cv. Chinese Spring showed 71% identity. Amount of identity between other sequences was low. Sequence comparison with NCBI dataset revealed identity between sequences of facultative genotype and cv. Chinese Spring with sequence of vrd1 gene in 2174 and Jugger cultivars (JQ915056.1 and JQ915055.1). Four conserved regions with low conservation coefficient were identified in the analyzed sequences.

Using Intr1/D/F-Intr1/D/R4 primer pair, a 997 bp fragment was amplified and sequenced in spring and facultative landraces and cv. thatcher. There was 70% identity between sequences spring landrace and Thatcher. Although there were differences between the analyzed sequences, a region of 435 bp with high identity and only few SNPs was identified. The sequences were blasted in NCBI databases and high identity (96%-99%) was observed between them and sequence of *VRN-D1* gene from *Triticum aestivum*, *Aegilop stauschii* and *Triticum spelta* (AY747606.1, AY747605.1, AB630964.1, AB630963.1, AB630961.1, AB630962.1 and KF800714.1).

A region of 571 bp conserved region with coefficient of 0.93 was also identified.

Information about the *Vrn* gene is important in considering the frost tolerance and low temperature response of cereals. The occurrence of *Vrn* alleles has been described for many wheat cultivars from different regions of the world. In this study, we characterized the genotype of *VRN-1* first intron in spring and winter Iranian wheat landraces. Stelmakh (1987) mentioned thahexaploid wheat genotypes with winter growth habit are homozygous for the recessive alleles at the three *VRN-1* loci. However, it was shown that in some cases the dominant *Vrn-B1* or *Vrn-D1* allele is not sufficient to determine spring growth habit and plants require low temperature condition, which is in case of facultative type (Sun *et al.*, 2009). The main factor determining flowering initiation is the level of the *VRN-1* transcript (Distelfeld *et al.*, 2009; Dhillon *et al.*, 2010). Sometimes the transcript level probably is too low despite the occurrence of the dominant *Vrn* allele, and this inhibits flowering.

Fu *et al.* (2005) characterized the *VRN-1* locus in 117 spring wheat cultivars from Argentina and California. The dominant *Vrn-A1* and *Vrn-D1* alleles were in ~56.5% in ~42% of the genotypes, respectively, regardless of regions of origin. The frequency of the *Vrn-B1* allele for cultivars from Argentina and California amounted to 66.1%, and 49.1%, respectively. The most common allelic combination was *Vrn-A1*, *Vrn-B1*, and *Vrn-D1*, which observed in 48.4% of the studied cultivars. Stelmakh (1987) did similar study in 45 wheat cultivars from the USA and Canada and dominant *Vrn-A1* allele was presented in 91.1%, *Vrn-B1* in 60%, and *Vrn-D1* only in 6.7% of the studied cultivars. Among the VRN alleles detected in Iranian wheat landraces, *VRN-A1* showed the lowest frequency (8.86%), followed by spring habit *Vrn-D1* (57.21%), dominant *Vrn-B1* (38.48%), and winter habit *vrn-D1* (32.15%).

Since *VRN1* is probably the primary target of vernalization and is essential for flowering, the characterization of the regulatory regions identified in the promoter and the first intron is critical to understand the regulation of flowering initiation in the temperate cereals (Distelfeld *et al.,* 2009). The importance of the *VRN1* first intron in maintaining low levels of *VRN1* transcripts before vernalization is supported by the discovery of at least 15 independent deletions and two repetitive elements insertions in this region associated with high levels of *VRN1* transcripts and spring growth habit in both wheat and barley (Fu *et al.,* 2005; Dubcovsky *et al.,* 2006; Cockram *et al.,* 2007). These indels may eliminate a binding site for a putative repressor that is down regulated by vernalization or affect a site required to establish a repressed state of the intron one chromatin (Fu *et al.,* 2005).

Fu *et al.* (2005) defined a 4.2-kb region of *Vrn1* intron-1 as the vernalization "critical region". This region is defined by the deletions that have a most distal start point (*Vrn-A1c* of IL369) and most proximal end (the 3? end of the *Vrn-D1a* deletion), within which deletions result in decreased vernalization sensitivity. It has been hypothesized that the "critical region" contains a binding site for a putative repressor that is down regulated by vernalization or encompass a region implicated in maintaining chromatin in a repressive state.

Muterko *et al.* (2014) analyzed sequence of the promoter area and first intron of *Vrn-D1* in 77 hexaploid accessions, representing five *Triticum* species sampled from different ecogeographic areas within 35 countries. They amplified a 1671-bp fragment in 7 accessions using Intr1/D/F-Intr1/D/R3 primer pair and a 997-bp fragment in 66 accessions and a novel allele of 1841-bp size in 4 accessions using Intr1/D/F-Intr1/D/R4 primer pair. Sequence alignment showed this novel variant includes an 844-bp insertion in *Vrn-D1* intron-1 (position +1,044; from 859 to 1,702-bp, counting from the start of intron-1 in Triple Dirk C (GenBank accession AY747606)). They detected some SNPs among the sequences and found identity between the amplified sequences and *Vrn-D1* reference sequences (GenBank: AB630962, AB630964, and AY616454). Fu et al (2005) in a study for survey large deletions within the first intron in *VRN-1* observed 98% identity between *VRN-A1* sequences in *T. monococcum* (13,465-bp) with 227 unique SNPs. They also reported a large deletion (1375-bp) in *VRN-A1* intron 1 in the four polyploid accessions relative to *T. monococcum*.

We detected identity between sequences of our genotypes with the *vrd1* gene. At least two genes with different expressiveness that control differences in winter wheat with respect to the length of the vernalization period have been identified in winter wheat. The symbol *vrd* (from the initial letters of the English words, vernalization requirement duration) was proposed to denote these genes following preliminary consultation with R.A. McIntosh (Stelmakh *et al.,* 2005). The genes are referred to by the notations *vrd1* and *vrd2*. The *vrd1* gene has been localized in chromosome 4A and the *Vrd2* gene in chromosome 5D (Fayt *et al.,* 2007).

In our study, were identified SNPs in some sequences after alignment and high identity among them. Also we observed identity between sequences and *VrnA1*, *VrnB1* and *VrnD1* GenBank sequences. Conserved regions were found during the sequences. These regions in majority of fragments had high conservation coefficient.

ACKNOWLEDGEMENTS

This research was supported by grants from the Center of Excellence in Cereal Molecular Breeding, University of Tabriz, Tabriz, Iran. The seed of Iranian wheat landraces was generously provided by the International Maize and Wheat Improvement Center (CIMMYT).

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