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## Allelic variation at the *VRN-1* promoter region in polyploid wheat

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**Abstract** Vernalization, the requirement of a long exposure to low temperatures to induce flowering, is an essential adaptation of plants to cold winters. We have shown recently that the vernalization gene *VRN-1* from diploid wheat *Triticum monococcum* is the meristem identity gene *APETALA1*, and that deletions in its promoter were associated with spring growth habit. In this study, we characterized the allelic variation at the *VRN-1* promoter region in polyploid wheat. The *Vrn-A1a* allele has a duplication including the promoter region. Each copy has similar foldback elements inserted at the same location and is flanked by identical host direct duplications (HDD). This allele was found in more than half of the hexaploid varieties but not among the tetraploid lines analyzed here. The *Vrn-A1b* allele has two mutations in the HDD region and a 20-bp deletion in the 5' UTR compared with the winter allele. The *Vrn-A1b* allele was found in both tetraploid and hexaploid accessions but at a relatively low frequency. Among the tetraploid wheat accessions, we found two additional alleles with 32 bp and 54 bp deletions that included the HDD region. We found no size polymorphisms in the promoter region among the winter wheat varieties. The dominant *Vrn-A1* allele from two spring

varieties from Afghanistan and Egypt (*Vrn-A1c* allele) and all the dominant *Vrn-B1* and *Vrn-D1* alleles included in this study showed no differences from their respective recessive alleles in promoter sequences. Based on these results, we concluded that the *VRN-1* genes should have additional regulatory sites outside the promoter region studied here.

### Introduction

The adaptability of common wheat (*Triticum aestivum* L.,  $2n=42$ , genomes AABBDD) to a large range of environments is partially due to the exploitation of genetic variation in vernalization requirement and day length for the control of ear emergence. Vernalization is the requirement of a long exposure to low temperatures to induce flowering and is essential to protect the sensitive flower organs from the cold.

Vernalization requirement in common wheat is mainly controlled by three orthologous *VRN-1* genes located in the middle of the long arms of chromosomes 5A, 5B, and 5D (Barrett et al. 2002; Dubcovsky et al. 1998; Galiba et al. 1995; Iwaki et al. 2002; Law et al. 1975; McIntosh et al. 2003; Nelson et al. 1995; Unrau 1950). The *VRN-A1*, *VRN-B1*, and *VRN-D1* genes are dominant for spring growth habit and epistatic to the alleles for winter growth habit. Therefore, winter cultivars are homozygous for the recessive alleles at the three *VRN-1* loci (Stelmakh 1987). A few wheat varieties have different vernalization genes (*VRN-B4* and *VRN-D5*) that are not linked with the *VRN-1* genes (Goncharov 2003; Kato et al. 2003; Law 1966). These additional vernalization genes are also dominant for spring growth habit.

A vernalization gene with a dominant winter growth habit (*VRN-2*) was described in diploid wheat (Dubcovsky et al. 1998; Tranquilli and Dubcovsky 2000; Yan et al. 2004). Although allelic variation for growth habit at the *VRN-2* locus has not been described yet in common wheat, downregulation of this gene by RNA interference in winter common wheat variety Jagger accelerated

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flowering more than one month (Yan et al. 2004). This result demonstrated that *VRN-2* also plays an important role in the regulation of vernalization in common wheat.

Multiple alleles have been described within the *VRN-1* locus, based on their different effects on vernalization requirement and flowering time (Koval and Goncharov 1998; Roberts and McDonald 1984; Tsunewaki and Jenkins 1961). However, the precise characterization of these multiple alleles has been complicated by the polyploid nature of common wheat and the absence of precise molecular information about the *VRN-1* gene.

We have recently used diploid wheat *T. monococcum* ( $2n = 14, A^m A^m$ ) as a simpler model to clone the *VRN-1* vernalization gene by a positional cloning approach (Yan et al. 2003). We demonstrated that *VRN-1* is the wheat orthologue of the *Arabidopsis* meristem identity gene *APETALA1* (*API*), and that it was upregulated by vernalization in both leaves and apices in the winter wheat accessions (Yan et al. 2003). More recently, a similar upregulation of *VRN-1* transcription by vernalization in the leaves was reported in common wheat (Danyluk et al. 2003; Trevaskis et al. 2003).

In *T. monococcum*, deletions in the *VRN-1* promoter region were linked with the dominant spring growth habit (Dubcovsky and Yan 2003; Yan et al. 2003). Therefore, we decided to study the orthologous regions in polyploid wheat to test if similar polymorphisms were linked to the spring growth habit in the polyploid species. In this study, we compared the promoters of the *VRN-A1*, *VRN-B1*, and *VRN-D1* genes from accessions carrying contrasting alleles for winter and spring growth habit.

## Materials and methods

### Plant materials

Dr. Kim Kidwell (Washington State University, USA) provided seeds of the isogenic lines of Triple Dirk originally developed by Pugsley (1971, 1972). Triple Dirk C (TDC) has a winter growth habit and recessive alleles at the three *VRN-1* genes (*vrnA1vrnB1vrnD1*). The other three isogenic lines have a spring growth habit determined by one dominant *Vrn-1* allele in each line. Triple Dirk D (TDD) has the *Vrn-A1* allele (*VrnA1vrnB1vrnD1*), Triple Dirk B (TDB) the *Vrn-B1* allele (*vrnA1VrnB1vrnD1*), and Triple Dirk E (TDE) the *Vrn-D1* allele (*vrnA1vrnB1VrnD1*). Dr. C. Qualset (Calif., USA) provided seeds for the isogenic winter lines of the spring varieties Anza and Yecora Rojo, developed by backcrossing the winter allele from the variety Phoenix into Winter-Anza and Winter-Yecora Rojo.

Based on the genetic data from Iwaki et al. (2000, 2001) and from the Catalogue of Wheat Gene Symbols (McIntosh et al. 2003), we selected additional common wheat lines with known dominant alleles for spring growth habit at the *VRN-A1* (26 accessions), *VRN-B1* (three accessions), and *VRN-D1* (four accessions) loci for sequencing (Table 1). Recessive *vrn-A1* alleles were sequenced from three additional winter varieties (Table 1). Growth habit was confirmed by growing these accessions in the greenhouse at 20–25°C under a long photoperiod (16 h light) and without vernalization. An additional 200 hexaploid wheat lines (68 winter and 132 spring)

**Table 1** Accessions with known *VRN-1* alleles

Habit	Allele	Origin of varieties, breeding lines and land races (LR)
Winter	<i>vrn-A1</i>	Australia and New Zealand: Triple Dirk C <sup>a</sup> . US: Cheyenne, Madsen, Winter-Anza. China: Chinese Spring
Spring	<i>Vrn-A1a</i>	Australia and New Zealand: Triple Dirk D <sup>a, i</sup> , Takari (PI 483058) <sup>b, j</sup> , Falcon (PI 292578) <sup>a</sup> . Armenia: LR (IL 187) <sup>c, j</sup> . Canada: Reward (PI 351819) <sup>a, j</sup> . Georgia: Hulugo (CP 51) <sup>d</sup> , LR (IL 378) <sup>c, j</sup> . Germany: Koga II (PI 232875) <sup>d</sup> . Iran: LR (IL 333) <sup>c, j</sup> . Japan: Konosu-25 (SS 2) <sup>b</sup> , Saitama 27 (PI 155279) <sup>c</sup> . Kazakhstan: Shortandinka (PI 326303) <sup>a, j</sup> . Sweden: LR (SL 33) <sup>c</sup> , LR (SL 39) <sup>c, j</sup> , Diamant II (PI 181467) <sup>f</sup> . Turkey: LR (IL 43) <sup>c, j</sup> . US: Hope (PI 192612) <sup>b, j</sup> , Thatcher (PI 168659) <sup>a, j</sup> , Anza <sup>i</sup> , Cadet (Citr 12053) <sup>g</sup>
Spring	<i>Vrn-A1b</i>	Canada: Marquis (PI 94548) <sup>h, k</sup> . Ethiopia: LR (IL 63, IL 66) <sup>c</sup> . Italy: LR (GR 46) <sup>c</sup> . Greece: LR (IL 12, IL 425) <sup>c</sup>
Spring	<i>Vrn-A1c</i>	Afghanistan: LR (IL 369) <sup>c</sup> . Egypt: LR (IL 162) <sup>c</sup>
Winter	<i>vrn-B1</i>	Australia and New Zealand: Triple Dirk C <sup>a</sup>
Spring	<i>Vrn-B1</i>	Australia and New Zealand: Triple Dirk B <sup>a</sup> , Festiguay (PI 330957) <sup>a</sup> . Italy: Mara (PI 244854) <sup>d</sup> . Russia: Mil'turum 321 (PI 155125) <sup>i</sup>
Winter	<i>vrn-D1</i>	Australia and New Zealand: Triple Dirk C <sup>a</sup>
Spring	<i>Vrn-D1</i>	Australia and New Zealand: Triple Dirk E <sup>a</sup> . China: Chinese Spring <sup>a</sup> Japan: Norin 61 (PI 235236) <sup>c</sup> , Ushio Komugi (PI 384010) <sup>e</sup> , Shinchunaga (PI 197128) <sup>e</sup>

<sup>a</sup>(Pugsley 1971, 1972)

<sup>b</sup>(Stelmakh 1987)

<sup>c</sup>(Iwaki et al. 2000, 2001)

<sup>d</sup>(Xin et al. 1988)

<sup>e</sup>(Gotoh 1979)

<sup>f</sup>(Maystrenko 1980)

<sup>g</sup>(Roberts and Larson 1985)

<sup>h</sup>(McIntosh et al. 2003)

<sup>i</sup>The two copies of the duplicated promoter region were sequenced

<sup>j</sup>Only the copy with the 131-bp foldback element was sequenced

<sup>k</sup>Two accessions classified as Marquis from Japan (SS8) and California showed the *Vrn-A1a* allele

**Table 2** Growth habit, geographic origin, *Vrn-A1* allele based on PCR assay, and names of the winter (*vrn-A1*) and spring lines (previously unknown *Vrn-A1* alleles)

Habit	Allele	Origin of analyzed varieties, breeding lines and land races
Winter	<i>vrn-A1</i>	Argentina: Klein 32, General Roca, Klein Rendidor, Prointa Puntal, Prointa Súper. Australia and New Zealand: Festival, Triple Dirk C. Belgium: Val. Chile: PI436202, Temu. France: Camp Remy, Chopin, Forby, Milpain, VPM1, Cappelle-Desprez. Germany: Tenor. Japan: Akakawa aka, Chihokukomugi, Fukuotome, Hayakomugi, Iwaino Daichi. Korea: Suweon 235. Russia: Bezostaja-1. US: Andrews, Barbee, Bonne Ville, Colt, Comanche, Eltan, Garland, Gene, Hill 81, Hoff, Hyak, Kmor, Lambert, Mac Vicar, Madsen, Malcolm, Meridian, Newton, Nugaines, OR900438, R901966, OR943663, Phoenix, PI518789, PI518796, PI518798, PI519290, Promontory, Red Chief, Rod, Rohde, Rulo, Stephens, Winter-Tanori, Tascosa, Tres, Triumph 64, Turkey, Yamhill, Winter Yecora Rojo. UK: Armada, Mercia. Yugoslavia: Sava, Rannaya
Spring	<i>Vrn-A1a</i> (insertion)	Argentina: ACA 302, ACA 601, Bonaerense Pasuco, Buck Arriero, Buck Biguá, Buck Brasil, Buck Catriel, Buck Chambergo, Buck Guaraní, Buck Guatimozín, Buck Manantial, Buck Mataco, Buck Namuncurá, Buck Nandú, Buck Palenque, Buck Patacón, Buck Pingo, Buck Yapeyú, Cooperación Liquén, Cooperación Millán, Diamante INTA, Granero INTA, Klein Chajá, Klein Flecha, Las Rosas INTA, Leones INTA, Pampa INTA, Prointa Bonaerense Alazán, Prointa Bonaerense Redomón, Prointa Colibrí, Prointa Elite, Prointa Granar, Prointa Imperial, Prointa Molinero, Zorzal. Canada: Canthatch. Mexico: Siete Cerros 66, Sonora 64, Yaqui 54. Uruguay: INIA Tijereta. US: Blanca, Brooks, Calorwa, Challenger, Choteau, Copper, Dirkwin, Hi-line, Klasic, Len, Marshall, Outlook, Owens, Poco Red, Pomerelle, Probrand 755, Rich, Serra, Spillman, Stoa, Sunstar II, Sunstar Promise, Treasure, Twin, UC1110, UC020380383, UC896, Waduel 94, Wawawai, Westbred 926, Yecora Rojo, Yolo
Spring	<i>Vrn-A1b</i> (deletion)	Argentina: Barleta, Klein Cobre, Pergamino Gaboto, Sinvalocho. US Big Club 60, Centennial, Hank, Scholar
Spring	Unknown <sup>a</sup>	Argentina: ACA 303, Bonaerense INTA Payador, Bonaerense INTA Potrillo, Bordenave Puan SAG, Buck Charrúa, Buck Farol, Buck Guapo, Buck Pampero, Buck Poncho, Buck Quequén, Buck Sureño, Buck Yatasto, Klein Don Enrique, Klein Escorpión, Klein Escudo, Klein Estrella, Klein Jabalí, Klein Martillo, Klein Pegaso, Klein Petiso, Klein Proteo, Klein Sagitario, Klein Salado, Marcos Juárez INTA, Prointa Gaucho. Mexico: Cajeme 71, Nainari 60, Pitic 62, Prointa Oasis. Uruguay: INIA Churrinche. US: Alpowa, Amidon, Clear White, Express, Inia 66R, Kern, Newana, Poso 48, Probred, Ramona50, RS15, Shasta, Tadinia, Tammy, UC1037, UC1041, UC1107, UC1128, UC1358, Vanna, Westbred 911, Whitebird

<sup>a</sup>PCR amplification of the same size as the recessive *vrn-A1* from Triple Dirk C

**Table 3** Accessions of tetraploid wheat lines with a spring growth habit

Species	Allele	Varieties, breeding lines and land races <sup>a</sup>
<i>Triticum turgidum</i> ssp. <i>durum</i>	<i>Vrn-A1b</i>	US: <u>ST 36</u>
<i>T. turgidum</i> ssp. <i>dicoccoides</i>	<i>Vrn-A1d</i>	Israel: <u>FA 15</u> , <u>Tabigha 15</u> , <u>Amrim 34</u> , Iraq: <u>Iraq 8736</u>
<i>T. turgidum</i> ssp. <i>dicoccum</i>	<i>Vrn-A1e</i>	Unknown origin: <u>ST 27</u> (= Vernal)
<i>T. turgidum</i> ssp. <i>durum</i>	Unknown <sup>b</sup>	Greece: Minos. Mexico: Altar 84, Carcomun, Mexicali 75. US: Aldura, Aruba, Duraking, Durex, Durfort, Eddie, Imperial, <u>Langdon</u> , Leeds, Ocotillo, <u>Reva</u> , <u>Ria</u> , Westbred 881, Westbred Laker, Westbred Turbo

<sup>a</sup>Underlined accessions were sequenced. No genetic information is available for these accessions

<sup>b</sup>PCR amplification of the same size as the recessive *vrn-A1* allele from Triple Dirk C

(Table 2) and 25 tetraploid spring lines (Table 3) were screened by PCR to assess the frequency of insertions and deletions in the *VRN-A1* promoter region. The *VRN-I* alleles present in these spring varieties were unknown.

Nulli-tetrasomic lines of ‘Chinese Spring’, N5AT5D, N5BT5D, and N5DT5B, were used to assign the BACs selected with the *VRN-I* gene to the A, B, and D genomes, and to validate the genome-specific primers.

#### BAC libraries

To obtain the sequences of the *VRN-I* genes from the A and B genomes, the 5× genome coverage BAC library of tetraploid durum wheat variety Langdon (Cenci et al. 2003) was screened by hybridization, using the last five exons of the *VRN-A<sup>m</sup>I* gene as a probe. This region excluded the conserved MADS-box to avoid selection of other members of the large family of MADS-box transcription factors. The same probe was used to screen a partial *EcoRI* BAC library of *T. tauschii*, the diploid donor of the D genome of hexaploid wheat (J. Dvorak, M.C. Luo, and H.B. Zhang, unpublished).

## Amplification and cloning

The promoter region of *T. monococcum VRN-A<sup>m</sup>1* gene (AY188333) provided the starting point to obtain the sequences from the polyploid species (Yan et al. 2003). Primers VRN1-1000F (5'-TGATGGATGTCTGG-TCGGTA-3') and VRN1-INT1R (5'-GCAGGAAATC-GAAATCGAAG-3') were designed based on the AY188333 sequence covering 1,000 bp upstream from the start codon, the first exon (185 bp), and 64 bp of the first intron. These primers were used to amplify and sequence the orthologous regions from the BAC clones from the three different genomes. The A, B, and D genome sequences were compared, and genome-specific forward primers were designed by including inter-genome polymorphisms within the 3' regions of the primers. Specific forward primers for the A, B, and D genomes were VRN1AF (5'-GAAAGGAAAATTCTGCTCG-3'), VRN1BF (5'-CAGTACCCCTGCTACCAGTG-3'), and VRN1DF (5'-CGACCCGGGCGGCACGAGTG-3'), respectively. These genome-specific primers were combined with the same degenerate reverse primer VRN1R, located within the first 20 bp of *VRN-1* exon 1 (5'-TGCACCTTCCC<sup>(C/G)</sup>CGCCCCAT-3'). This degenerate primer was used to accommodate a polymorphism between the *VRN-1* gene in the A genome ("C") and the B and D genomes ("G"). Annealing temperatures for the PCR amplifications using the genome-specific primers were 55°C (VRN1AF), 58°C (VRN1BF), and 60°C (VRN1DF).

PCR products were purified by PCR Wizard columns (Promega) and either sequenced directly or cloned into pCR 4-TOPO TA vectors (Invitrogen) and then sequenced using M13 primers. Sequencing was performed in an ABI3730.

## Results

### Genome-specific primers

Twelve positive BAC clones were identified in the screening of the tetraploid BAC library of Langdon (Cenci et al. 2003). These BACs were assembled into two groups by *Hind*III fingerprints, which were further validated by hybridization of their Southern blots with the

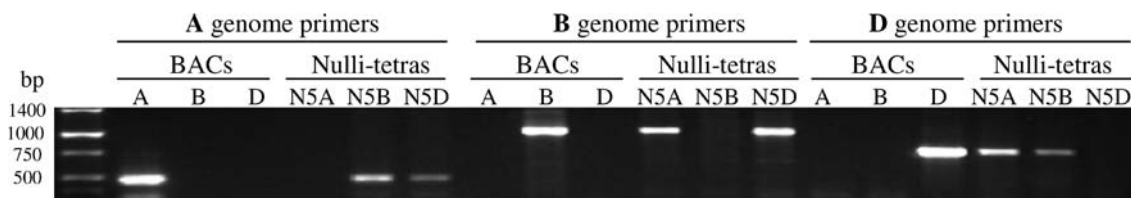
VRN-A1 probe. This result indicated that only two copies of the *VRN-1* gene were present in tetraploid wheat Langdon. Langdon BACs 1256C17 and 1225D16 were selected to represent each of the two different groups. BAC clone 22 J2 was selected from a partial *Eco*RI BAC library of *T. tauschii*, representing the D genome.

Sequences from 1 kb upstream from the start codon to 63–64 bp in intron 1 were obtained from BACs 1256C17 (GenBank AY616452), 1225D16 (GenBank AY616453), and BAC 22 J2 (GenBank AY616454), using primers VRN1-1000F and VRN1-INT1R. Comparisons among these sequences revealed several differences among the three genomes that were used to design genome-specific primers VRN1AF, VRN1BF, and VRN1DF. The sequence from BAC 1256C17 was more similar to the A<sup>m</sup> genome of *T. monococcum* than the sequence from BAC 1225D16, indicating that 1256C17 was likely from the A genome of Langdon.

Specificity of the three genome-specific primers was confirmed by PCR, using DNA from the three different BACs and genomic DNA from nulli-tetrasomic lines of 'Chinese Spring' N5AT5D, N5BT5D, and N5DT5B (Fig. 1). As an example, primers VRN1AF/VRN1R amplified a PCR product in the reactions including DNA from BAC 1256C17, but not in those with DNA from BACs 1225D16 or 22 J2. In addition, this primer pair produced the expected PCR product with DNA from N5BT5D and N5DT5B but not from N5AT5D (Fig. 1). This analysis confirmed that BAC 1256C17 was from chromosome 5A and BAC 1225D16 from chromosome 5B.

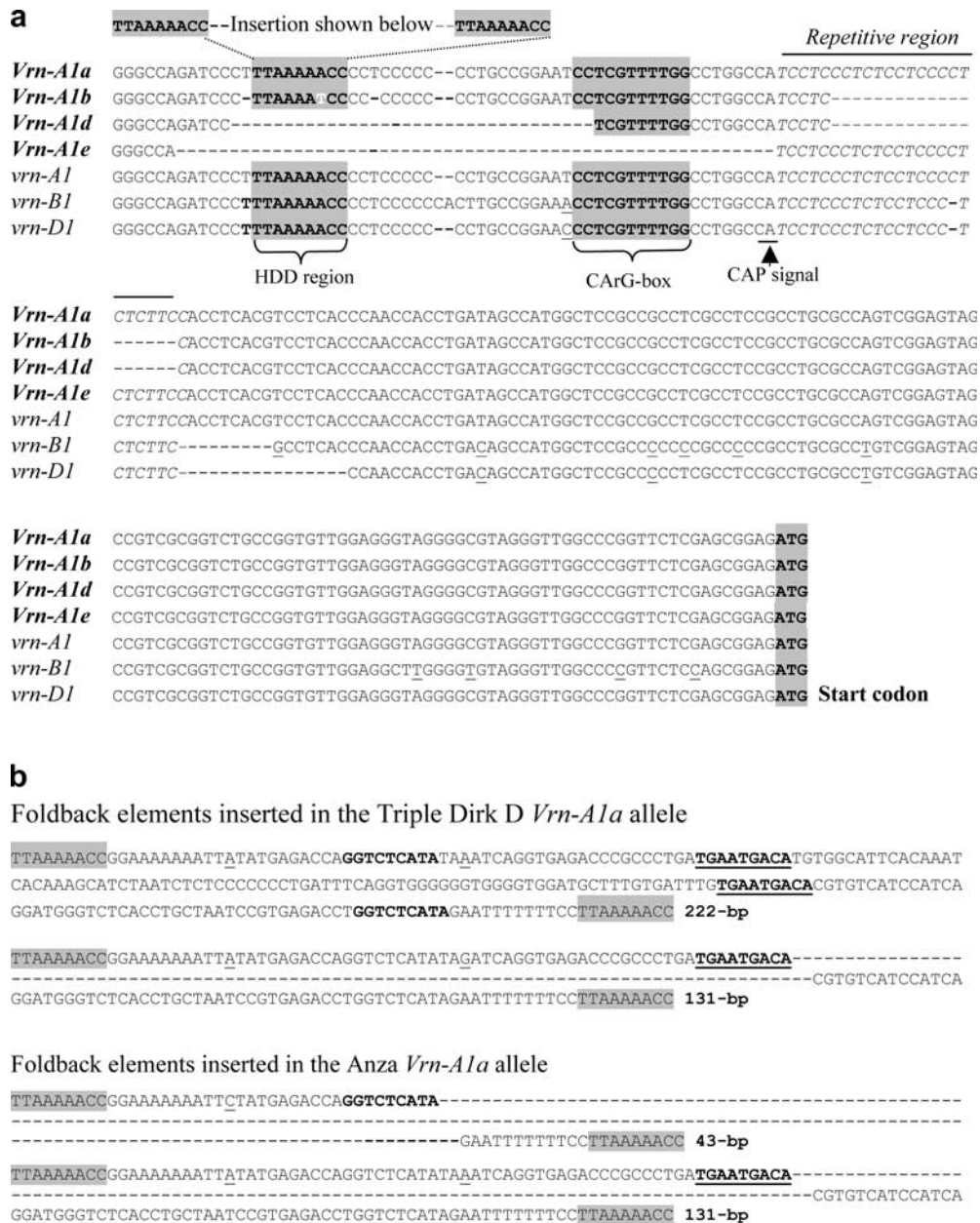
### *VRN-1* promoter regions from winter wheat varieties (*vrn-1* alleles)

The genome-specific primers were used to amplify and sequence the promoter and 5' UTR regions of the recessive *vrn-A1* (GenBank AY616455), *vrn-B1* (GenBank AY616456), and *vrn-D1* (GenBank AY616457) alleles from winter wheat TDC (Fig. 2a). The approximate location of the initiation of transcription was inferred by comparing the genomic sequences with available ESTs from each of the three *VRN-1* genes. Based on comparisons with ESTs CA599270 (*VRN-A1*),



**Fig. 1** Validation of genome-specific primer pairs VRN1AF/VRN1R (A genome), VRN1BF/VRN1R (B genome), VRN1DF/VRN1R (D genome). A, B, and D indicate PCR products from BACs from the A genome (Langdon BAC 1256C17), B genome

(Langdon BAC 1225D16), and D genome (*Triticum tauschii* BAC 22 J2). Primers were also tested in nullitetrasomic lines missing each of the three homoeologous group 5 chromosomes, N5AT5D, N5BT5D, and N5DT5B



**Fig. 2 a** Section of the sequence alignment of the *VRN-1* promoter amplified with genome-specific primers VRN1AF/VRN1R. Recessive *vrn-A1*, *vrn-B1*, and *vrn-D1* alleles were obtained from the winter Triple Dirk C (TDC) line. The *Vrn-A1a* sequence [Triple Dirk D (TDD) and Anza] includes a duplication of the promoter region. The *top line* (*Vrn-A1a*) indicates the foldback element inserted into the promoter region (the remaining sequence is identical to the *vrn-A1* recessive allele). *Vrn-A1b*: Marquis, IL12, IL425. The *Vrn-A1c* sequence is not included because it is identical to *vrn-A1*. *Vrn-A1d*: *T. dicoccoides* FA15-3 (spring), *Vrn-A1e*:

*T. dicoccom* ST27 (spring). The putative CAP signal for transcriptional initiation is indicated by an *arrow*. A TC-rich repetitive region of the 5' UTR is in *italics*. Deletions are indicated by *dashes*. Polymorphic bases are *underlined*. The ATG start codon is indicated in *gray*. The 9-bp host direct duplication and the putative CArG box are *highlighted in gray*. **b** Duplicated foldback element in TDC and Anza. Two 9-bp direct repeats flanking the deletions in the foldback elements are indicated in *boldface and underlined* in the longest 222-bp element

CA593340 (*VRN-B1*), and CD936729 (*VRN-D1*), the initiation of transcription was located approximately between -162 bp and -166 bp from the start codon (Fig. 2a).

A variable TC-rich repetitive region was found at the start of the *VRN-1* 5' UTR. Within this region, the *vrn-B1* and *vrn-D1* alleles shared a 1-bp deletion and the

start of a second deletion that extended for 8 bp in the *vrn-B1* genome and 15 bp in the *vrn-it D1* loci. A putative CArG box (a recognition sequence for MADS-box proteins) was found in the promoter region a few base pairs upstream from the predicted sites of transcription initiation (Yan et al. 2003). This site was conserved among the three genomes (Fig. 2). The first base

upstream from the CArG box was variable among genomes, suggesting that it is not essential for its function (Fig. 2a).

Approximately 43–45 bp upstream from the putative site of transcription initiation, a putative TATA box (TTTAAAA) was conserved among the recessive alleles (Fig. 2a).

#### Allelic variation at the *VRN-1* promoter region in accessions with known alleles

No sequence differences were found between the dominant *Vrn-B1* allele from TDB and the recessive *vrn-B1* allele from TDC in the first 591 bp upstream from the start codon. Partial sequences of the *VRN-B1* promoter region ( $\approx 400$  bp including the CArG box and the putative TATA-box region) were also obtained from four hexaploid varieties known to have the *Vrn-B1* dominant allele (Table 1, PI 155125, PI 168661, PI 244854, and PI 330957; McIntosh et al. 2003). These sequences showed no differences with the *VRN-B1* promoter sequences from TDB and TDC. Comparison of the *VRN-B1* sequence from hexaploid wheat (GenBank AY616456) with that from the B genome BAC from Langdon (GenBank AY616453) revealed no polymorphism.

Similarly, no sequence differences in the promoter region were found between 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 (GenBank AY616457). Partial sequences of the *VRN-D1* promoter region ( $\approx 400$  bp) obtained from three additional hexaploid varieties carrying the *Vrn-D1* dominant allele (Table 1, PI 235236, PI 384010 and PI 197128; McIntosh et al. 2003) showed no differences with the recessive *vrn-D1* allele from TDC. Only one base difference (positions–707 from the start codon) was found between the sequences obtained from the *VRN-D1* promoter region of hexaploid wheat and the sequence obtained from the D genome of *T. tauschii* (GenBank AY616454).

Amplification of genomic DNA from the promoter region of the dominant *Vrn-A1* allele from TDD, using primers VRN1AF and VRN1-INT1R, showed the presence of PCR products of two different lengths, both of them larger than the PCR product of the *vrn-A1* allele. Sequencing of these two fragments confirmed that the promoter region of the *Vrn-A1* allele was duplicated in the TDD genome. This allele was designated *Vrn-A1a*. The simultaneous presence of both fragments in TDD and their absence in the isogenic line TDC suggested that these two fragments were linked.

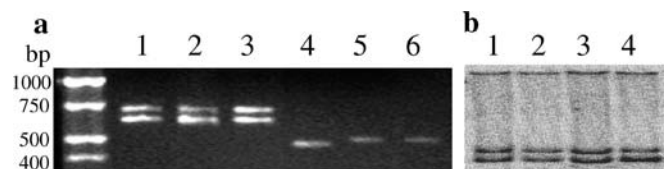
The two *Vrn-A1a* promoter fragments differed from the recessive *vrn-A1* allele from TDC by the insertion of a 222-bp foldback element in the larger fragment (GenBank AY616458) and a 131-bp foldback element in the smaller fragment (GenBank AY616459) (Fig. 2b). The two halves of the larger foldback element showed

good similarity along their complete length in inverted orientation. The smaller foldback element differed from the larger one by one SNP and one deletions of 91 bp. The deletion occurred between two 9-bp direct repeats (TGAATGACA, Fig. 2b). Both foldback elements were inserted in the same site and created the same 9-bp host direct duplication (HDD = TTAAAAACC). These results suggest that the duplication of the promoter region occurred after the insertion of the foldback element.

To determine the copy number of this foldback element, we hybridized four high-density filters of the Langdon BAC library (73,728 clones, 950 Mb, 0.73 genome coverage) with a probe including the 131-bp foldback element. The 275 positive BAC clones detected in these four filters suggested that approximately 375 copies of this foldback element are present in the tetraploid wheat genome. We named this foldback element “Spring” and deposited it in the Triticeae Repeat Sequence Database (TREP, <http://wheat.pw.usda.gov/ggpages/Repeats/index.shtml>).

In the spring isogenic line of Anza, the 222-bp foldback element was replaced by a 43-bp foldback element with the same insertion site and a 9-bp HDD (GenBank AY616460). The 43-bp foldback element likely originated from a 179-bp deletion that occurred within 9-bp direct repeats GGTCTCATA in the 222-bp foldback element. The 131-bp foldback element in Anza differed from the one in TDD only by one SNP (Fig. 2b). The amplification products including the 43-bp and 131-bp foldback elements were both present in Anza and in an additional isogenic spring line in Yecora Rojo but were absent in their respective winter lines, confirming the linkage between these two copies.

Analysis of the *Vrn-A1a* allele with primers VRN1AF and VRN1-INT1R demonstrated that the duplication extended at least 256 bp upstream from the insertion site of the foldback element to the first 64 bp of intron 1 (including a complete exon 1). The sequence flanking the foldback elements was identical between the dominant *Vrn-A1* and recessive *vrn-A1* alleles, suggesting a recent insertion time. To test if the complete *VRN-A1* gene was duplicated in the varieties carrying the duplicated promoter regions, we hybridized *EcoRV*-digested genomic DNA from TDD



**Fig. 3** a PCR amplification with primers VRN1AF/VRN1R. *Vrn-A1a* allele: duplicated promoter region carrying the 131-bp and 43-bp foldback elements. 1 Reward; 2 Thatcher; 3 Anza; 4 *Vrn-A1b* allele: 20-bp deletion, Marquis (PI 94548); 5 and 6 Recessive *vrn-1* alleles from TDC and Winter Anza. b Southern blot hybridized with a *VRN-1* probe including exons 4 to 8. 1 Triple Dirk D (*Vrn-A1a*), 2 TDC (*vrn-A1*), 3 Anza (*Vrn-A1a*), 4 Winter-Anza (*vrn-A1*)

(*Vrn-A1a*), TDC (*vrn-A1*), Anza (*Vrn-A1a*), and the isogenic line Winter-Anza (*vrn-A1*) with a probe including the *VRN-A1* region between exons 4 and 8. No additional hybridization fragments were detected in the isogenic lines carrying the *Vrn-A1a* allele. In addition, all the restriction fragments showed similar hybridization intensity in the lines with and without the promoter duplication, suggesting that this duplication did not include the complete *VRN-A1* gene (Fig. 3b).

Analysis of the *VRN-A1* promoter region from additional 26 lines previously known to have the dominant *Vrn-A1* alleles revealed the presence of the foldback element insertion and duplication in 18 of them (Table 1). Only the band carrying the 131-bp foldback was sequenced from these additional accessions.

Six out of the other eight accessions showed a smaller PCR amplification product than the one found in the recessive *vrn-A1* allele (Table 1). Sequencing of this PCR product revealed a 20-bp deletion in the TC-repetitive region of the 5' UTR, a 1-bp deletion adjacent to the HDD region, and a characteristic polymorphic T within the HDD region (Fig. 2). This allele, designated *Vrn-A1b* (GenBank AY616461), was found in the variety Marquis (PI94548) and in landraces from Greece (IL 12 and IL 425), Italy (GR 46), and Ethiopia (IL 63 and IL 66) (Table 1).

The promoter sequence from two landraces from Afghanistan (IL 369) and Egypt (IL 162), known to have the dominant *Vrn-A1* allele, was identical to the sequence of the *vrn-A1* allele from TDC. This allele was designated *Vrn-A1c*. The presence of the *Vrn-A1* allele in these two lines was inferred by the absence of winter plants in F<sub>2</sub> populations from the crosses between the tester line TDD carrying only the dominant *Vrn-A1* allele and IL 162 (145 F<sub>2</sub> plants analyzed) or IL 369 (84 F<sub>2</sub> plants analyzed). Plants with a winter growth habit were recovered from the crosses between these two lines and TDB but not from the cross with TDE, suggesting that these lines have dominant *Vrn-A1* and *Vrn-D1* alleles.

#### Allelic variation at the *VRN-A1* promoter region in common wheat accessions with unknown alleles

We found no sequence differences among the promoter regions of recessive *vrn-A1* alleles from five *T. aestivum* lines (Triple Dirk C, Winter-Anza, Chinese Spring, Madsen, and Cheyenne). In addition, we characterized by PCR a collection of 68 winter varieties of common wheat from different regions of the world. The PCR products obtained with the A-genome-specific primers VRN1AF/VRN1R were all of the same size as the recessive *vrn-A1* allele from TDC (Table 2), indicating that none of these lines has the *Vrn-A1a* or *Vrn-A1b* alleles.

In contrast, the PCR survey of 132 spring varieties with unknown *VRN-I* alleles showed that 55% of them have the foldback element insertion (*Vrn-A1a* allele),

and 6% of them have deletions that could be detected in agarose gels (likely the *Vrn-A1b* allele). Among the spring wheat varieties included in this study that were released before 1970, the *Vrn-A1a* allele was detected only in one out of seven spring varieties from Argentina (Buck Manantial, 1965) and in none of the five early US varieties. During the same period, three out of the five studied varieties from CIMMYT that were released before 1970 (Yaqui 54, Sonora 64, and Siete Cerros 66) had the *Vrn-A1a* allele.

Fifty-three spring varieties included in this survey showed PCR products of similar size to those obtained from the recessive *vrn-A1* allele from TDC. Since no genetic studies were available for the vernalization response of these varieties, it was not possible to establish which *VRN* allele was responsible for their spring growth habit. Therefore, these varieties are listed as "Unknown" alleles in Table 2.

#### Allelic variation at the *VRN-I* promoter regions in tetraploid accessions with unknown alleles

We performed an additional PCR screening of the *VRN-A1* promoter region (primers VRN1AF/VRN1R) in 25 lines of tetraploid wheat to see if the alleles observed in common wheat were contributed by the tetraploid species. The *VRN* alleles present in these lines were unknown.

The PCR screening revealed the presence of shorter amplification products than those from the *vrn-A1* allele in one *T. turgidum* ssp. *durum* Husn., three *T. turgidum* ssp. *dicoccoides* Thell., and one *Triticum turgidum* ssp. *dicoccum* Schrank ex Schübler accessions. Sequencing of these accessions showed that *T. turgidum* ssp. *durum* accession ST 36 had the characteristic mutations and deletions of the *Vrn-A1b* allele previously described for hexaploid wheat (Fig. 2a). The three *T. turgidum* ssp. *dicoccoides* shared the same 32-bp deletion, which included the complete HDD region and part of the CArG box. This allele was designated *Vrn-A1d* (GenBank AY616462) (Fig. 2). A different 54-bp deletion, including the CArG box and HDD regions, was found in the *Vrn-A1* promoter region of *Triticum turgidum* ssp. *dicoccum* accession ST 27 (Fig. 2). This allele was designated *Vrn-A1e* (GenBank AY616463). Both the 32-bp deletion and the 54-bp deletion occurred between perfect direct repeats (ATCC and GGCCA, respectively). One of these repeats was eliminated in each of the deletions. The association between the *Vrn-A1d* and *Vrn-A1e* alleles with a dominant spring growth habit still needs experimental demonstration.

The other 19 accessions from *T. turgidum* ssp. *durum* analyzed in this study with primers VRN1AF/VRN1R showed amplification products of similar length to the recessive *vrn-A1* allele. These varieties are listed as "Unknown" alleles in Table 3. None of the tetraploid accessions included in this study showed the longer PCR products characteristic of the *Vrn-A1a* allele.



## Haplotype variation in other regions of the *VRN-A1* gene

In a previous study, we characterized the allelic variation of the *VRN-A1* gene within an 810-bp region between intron 4 and exon 8 in a set of common wheat lines including 40 spring and 37 winter lines (Sherman et al. 2004). Three diagnostic nucleotide polymorphisms defined two haplotypes, which were associated with the dominant (*s* haplotype) and recessive (*w* haplotype) *VRN-A1* alleles, in 75 out of the 77 varieties analyzed (Sherman et al. 2004).

We sequenced this 810-bp region in one accession from each of the different alleles discovered in the *VRN-A1* promoter region in common wheat. We found that the *Vrn-A1a* and *Vrn-A1b* alleles were associated with the *s* haplotype, whereas the two accessions with the *Vrn-A1c* allele were associated with the *w* haplotype for the distal part of the *VRN-A1* gene. Therefore, the molecular markers developed by Sherman et al. (2004) provide a valuable tool to select simultaneously for the two most frequent mutations at the *VRN-A1* promoter region associated with the spring growth habit.

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## Discussion

### Molecular diversity at the *Vrn-A1* promoter region

Hexaploid *T. aestivum* originated relatively recently ( $\approx 8,000$  years) from the hybridization of tetraploid *T. turgidum* and *T. tauschii* (Huang et al. 2002). This recent origin is reflected in a low level of polymorphism (approximately 1 in 1,000 bp) among common wheat varieties (Bryan et al. 1999). The *VRN-A1* promoter region revealed a relatively higher level of variation in the first 50 bp upstream from the transcription start (Fig. 2a). It is tempting to speculate that this relatively high level of sequence variation could be related to the strong selection pressure applied to this gene during the last 8,000 years of wheat domestication. Wheat has moved with the human populations to very different locations and has been selected to adjust its flowering time to contrasting environments. These different selection pressures might have favored the relatively high level of polymorphisms observed in this putative regulatory region.

The molecular diversity found in the *VRN-A1* promoter region parallels previous reports of allelic series within the vernalization loci (Koval and Goncharov 1998; Roberts and McDonald 1984; Tsunewaki and Jenkins 1961). The introgression of the different *VRN-A1* alleles in a common genetic background will be useful to test the correspondence between the molecular variation in the promoter region and the variation in flowering time or vernalization requirement. The size differences found in this study among the different *VRN-A1* alleles will provide a useful tool to accelerate the development of these isogenic lines.

These size differences among alleles were frequently generated by deletions flanked by short direct repeats. The 32-bp and 54-bp deletions in the *Vrn-A1d* and *Vrn-A1e* alleles and the deletions within the *Vrn-A1a* fold-back element were all flanked by perfect direct repeats. We made a similar observation in a comparison between orthologous sequences in wheat and barley (Ramakrishna et al. 2002). In the wheat–barley comparison, we did not know which sequence was ancestral and therefore, we could not distinguish between a deletion involving direct repeats and an insertion of a repetitive element generating HDD. Fortunately, in this case we know that the functional winter *vrn-A1* allele is the ancestral one, and that these four polymorphisms likely originated by deletions rather than by insertions of repetitive elements. These deletions can be generated by replication slippage, a mechanism that has been shown to produce deletions between short duplications in bacteria (Trinh and Sinden 1993), yeast (Tran et al. 1995), and mammalian mitochondria (Madsen et al. 1993). Our data suggest that this mechanism is also frequent in wheat.

The *Vrn-A1a* allele was particularly interesting, because it was present in more than half of the spring varieties released in the United States and Argentina between 1970 and 2004. This high frequency was not observed in the older varieties included in this study. Only one out of the 12 spring varieties released before 1970 in these two countries had the *Vrn-A1a* allele (8%). In contrast, three out of the five early varieties from CIMMYT had this allele. These results suggest that the increase in the frequency of the *Vrn-A1a* allele in the spring varieties from Argentina and the United States might be related to the introduction of the semi-dwarf germplasm from CIMMYT in the 1970s.

The two copies of the promoter of the *Vrn-A1a* allele, with their characteristic foldback elements, were found together in the spring forms of the three pairs of isogenic lines included in this study, suggesting that they are linked. However, the complete structure of this duplication is still not completely understood. The absence of sequence differences outside the inserted foldback element suggests that this insertion/duplication has a recent origin. It is possible that the insertion/duplication event occurred in hexaploid wheat, since none of the tetraploid accessions analyzed in this study showed the *Vrn-A1a* allele. However, a larger tetraploid collection should be screened to confirm this hypothesis. The presence of the *Vrn-A1b* allele in *T. turgidum* ssp. *durum* confirmed that this allele originated at the tetraploid level.

The presence of insertions or deletions in the *VRN-A1* promoter in most of the varieties previously classified as dominant *Vrn-A1* and their absence in the varieties classified as recessive *vrn-A1* provides additional evidence to the correspondence between the *VRN-A1* locus and the *API* gene (Yan et al. 2003).

## Regulatory regions in the *VRN-1* gene

In the high-density mapping population for the *VRN-A<sup>m</sup>1* locus in diploid wheat *T. monococcum*, the spring growth habit was completely linked to a 20-bp deletion in the promoter region adjacent to the CArG box (Yan et al. 2003). The CArG box is a common binding site for MADS-box proteins (Tilly et al. 1998), suggesting that this site might be involved in the regulation of *VRN-1* by interacting with other MADS-box proteins. A survey of the *VRN-A<sup>m</sup>1* promoter region in a collection of spring accessions of cultivated *T. monococcum* showed three additional deletions that affected the CArG box, suggesting that this was an important region in the regulation of the *VRN-A<sup>m</sup>1* gene (Dubcovsky and Yan 2003; Yan et al. 2003).

We initially thought that the CArG box might be a recognition site for the *VRN-2* gene, a dominant repressor of flowering that shows strong epistatic interactions with *VRN-A1* (Tranquilli and Dubcovsky 2000). In the presence of the recessive *vrn-2* allele, variation at the *VRN-A1* locus has no effect on vernalization requirement, suggesting that *VRN-2* is a repressor of *VRN-A1* (Yan et al. 2003). Mutations in the CArG box would block the recognition of the *VRN-2* repressor, resulting in a dominant spring growth habit. However, we have recently cloned the *VRN-2* gene and found that it is not a MADS-box gene (Yan et al. 2004), suggesting that *VRN-2* might not be the gene that directly interacts with the CArG box in the *VRN-A1* promoter.

Two of the deletions detected in the *VRN-A1* promoter region in tetraploid wheat species also included the CArG box (*Vrn-A1d* and *Vrn-A1e*, Fig. 2a). However, the sequence differences detected in the *Vrn-A1a* and *Vrn-A1b* alleles from the hexaploid varieties were not in the CArG box but in the close HDD region (Fig. 2a). The putative importance of the HDD region is highlighted by its conservation within the orthologous sequence in rice (AF377947, TTTAAAAT). The HDD region includes most of the TTTAAAA (A/T) sequence with good similarity to a TATA box (Bucher 1990). This region is located -44 bp from the start of transcription, within the range of other plant gene TATA boxes (Bucher 1990). However, it seems unlikely that this conserved region plays an essential role in the initiation of the *VRN-1* transcription, because transcript levels of the *VRN-A<sup>m</sup>1* gene in *T. monococcum* accession PI 349049, which has a 34-bp deletion encompassing the HDD region (Yan et al. 2003), were similar to those of vernalized winter *T. monococcum* accessions (A. Loukoianov and J. Dubcovsky, unpublished).

The strong association between spring growth habit and the presence of mutations in the promoter of the *VRN-A1* gene in hexaploid wheat varieties can be explained by two alternative hypotheses. The simpler hypothesis is that these mutations are sufficient to cause a spring growth habit. However, we can not rule out the alternative hypothesis that the spring growth habit was determined by mutations outside the *VRN-A1* promoter

region, and that the mutations observed in this study were selected within the spring germplasm by chance or because of their positive effect on the faster growing cycle required by these spring varieties.

Regulatory sites outside the promoter region have been reported in other MADS-box genes (He et al. 2003; Sung and Amasino 2004). In *Arabidopsis*, alterations in the methylation and de-acetylation of histone H3 in certain regions of the first intron of *FLC* are essential for establishing and maintaining a repressed status after vernalization. Regulatory regions within introns have been reported also for other genes (Kapranov et al. 2001; Wang et al. 2002). Therefore, it would be important to compare the complete sequences of the dominant and recessive *VRN-1* alleles, a task that will be complicated by the large size of the first intron (9,574 bp in *T. monococcum*) and the hexaploid nature of common wheat.

The absence of differences in the promoter sequences between the dominant *Vrn-A1c*, *Vrn-B1*, and *Vrn-D1* alleles and their respective recessive alleles supports the hypothesis that additional regulatory sites are present in these genes outside the promoter region analyzed in this study. A complex regulation of the *VRN-1* expression was expected, since this gene is responsible for the initiation of flowering and, therefore, has a significant impact on the survival of the species.

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