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Authors

Fu, Daolin
Szűcs, Péter
Yan, Liuling
et al.

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ORIGINAL PAPER

**Daolin Fu · Péter Szűcs · Liuling Yan
Marcelo Helguera · Jeffrey S. Skinner
Jarislav von Zitzewitz · Patrick M. Hayes
Jorge Dubcovsky**

Large deletions within the first intron in *VRN-1* are associated with spring growth habit in barley and wheat

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Abstract The broad adaptability of wheat and barley is in part attributable to their flexible growth habit, in that spring forms have recurrently evolved from the ancestral winter growth habit. In diploid wheat and barley growth habit is determined by allelic variation at the *VRN-1* and/or *VRN-2* loci, whereas in the polyploid wheat species it is determined primarily by allelic variation at *VRN-1*. Dominant *Vrn-A1* alleles for spring growth habit are frequently associated with mutations in the promoter region in diploid wheat and in the A genome of common wheat. However, several dominant *Vrn-A1*, *Vrn-B1*, *Vrn-D1* (common wheat) and *Vrn-H1* (barley) alleles show no polymorphisms in the promoter region relative to their respective recessive alleles. In this study, we sequenced the complete *VRN-1* gene from these accessions and found that all of them have large deletions within the first intron, which overlap in a 4-kb region. Furthermore, a 2.8-kb segment within the 4-kb region showed high sequence conservation among the different recessive alleles. PCR markers for these deletions showed that similar deletions were present in all the accessions with known *Vrn-B1* and *Vrn-D1* alleles, and in 51 hexaploid spring wheat accessions previously shown to have no polymorphisms in the *VRN-A1* promoter region. Twenty-four tetraploid wheat accessions had a similar deletion in *VRN-A1* intron 1. We hypothesize that the 2.8-kb conserved region includes regulatory elements important for the vernalization requirement. Epistatic interactions between *VRN-H2* and the *VRN-H1* allele with the intron 1 deletion suggest that the deleted region may include a recognition site for the flowering repression mediated by the product of the *VRN-H2* gene of barley.

Keywords Wheat · Barley · Vernalization · *VRN-1* · Allelic variation

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D. Fu · L. Yan · J. Dubcovsky (✉)
Department of Plant Sciences, University of California,
One Shields Av, Davis, CA 95616, USA

P. Szűcs
Agricultural Research Institute of the Hungarian Academy of Sciences,
2462 Martonvásár, Hungary

M. Helguera
EEA INTA Marcos Juárez,
CC 21, 2580 Marcos Juárez, Argentina

J. S. Skinner · J. von Zitzewitz · P. M. Hayes
Department of Crop and Soil Science and Horticulture,
Oregon State University, Corvallis, OR 97331, USA

Introduction

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. Vernalization is defined as "the acquisition or acceleration of the ability to flower by a chilling treatment" (Chouard 1960). The physiology of vernalization has been studied extensively in cereals, but only a few genes from this molecular pathway are currently known (Yan et al. 2003, 2004b).

In wheat and barley, the determination of the vernalization requirement involves an epistatic interaction between the genetic loci *VRN-1* and *VRN-2* (Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000). Positional cloning of these two genes from diploid wheat (*Triticum monococcum* L., 2n = 14, genome A^mA^m) revealed that *VRN-1* encodes a MADS-box transcription factor that is orthologous to the *Arabidopsis* meristem identity gene *APETALA1* (Yan et al. 2003), while the *VRN-2* gene codes for a zinc finger-CCT domain transcription factor with no clear orthologues in *Arabidopsis* or rice (Yan et al. 2004b). The *VRN-1* gene is dominant for the spring growth habit and it is up-regulated by vernalization in winter lines (Danyluk et al. 2003; Trevaskis et al. 2003; Yan et al. 2003), whereas the *VRN-2*

gene is dominant for the winter growth habit and is down-regulated by vernalization (Yan et al. 2004b). In the spring genotypes, transcription of *VRN-1* is independent of vernalization (Trevaskis et al. 2003; Yan et al. 2003).

Based on their transcription profiles, the sequence differences 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. 2004b) result in an inactive repressor. A single functional copy of the *VRN-2* gene is sufficient to repress flowering, explaining the dominant winter growth habit. The model also suggests that mutations that alter the *VRN-1* recognition site for the VRN-2 repressor are responsible for the dominant spring growth habit characteristic of the *VRN-1* locus.

In diploid wheat, deletions in the *VRN-A*^{m}*1* promoter region were found to be associated with the spring growth habit in high-density mapping populations (Yan et al. 2003). A survey of the same promoter region in the polyploid wheat species *T. turgidum* ssp. *durum* Husnot. (2n = 28, genomes AABB) and *T. aestivum* L. (2n = 42, genomes AABBDD) revealed that most common wheats and a few tetraploid wheats carrying the dominant *Vrn-A1* allele have insertions, deletions or mutations in the *Vrn-A1* promoter region (Dubcovsky and Yan 2003; Yan et al. 2004a). However, we also observed that the dominant *Vrn-A1* alleles from the tetraploid cultivar Langdon and the hexaploid Afghanistan land race IL369, as well as the dominant *Vrn-B1* and *Vrn-D1* alleles, did not differ from their respective recessive alleles in the promoter sequence (Yan et al. 2004a). A similar observation was made for the promoter region of the vernalization gene *VRN-H1* from barley (*Hordeum vulgare* subsp. *vulgare* L., 2n = 14, genomes HH). Based on these results we concluded that the *VRN-1* genes should have additional regulatory sites outside the promoter region (Yan et al. 2004a).

In this study, we report comparisons between dominant and recessive *VRN-1* alleles in barley and polyploid wheat, and demonstrate the existence of overlapping deletions in a region of intron 1. We discuss the implications of this discovery for our current model for the regulation of flowering time by vernalization in temperate cereals.

Materials and methods

Plant materials

Nearly isogenic lines (NILs) of Triple Dirk (Pugsley 1971, 1972), including Triple Dirk B (TDB), Triple Dirk C (TDC), Triple Dirk D (TDD) and Triple Dirk E (TDE), were provided by Dr. Kim Kidwell (Washington State University, Pullman, WA). TDC has a winter growth habit and carries recessive alleles at the three *VRN-1* loci (*vrnA1vrnB1vrnD1*). The other three Triple Dirk NILs have a spring growth habit determined by dominant *Vrn-A1* (TDD), *Vrn-B1* (TDB) and *Vrn-D1* (TDE) alleles. In addition, this study included eight accessions carrying the dominant *Vrn-B1* and six accessions carrying the *Vrn-D1* alleles (Table 1), as determined by previously published genetic studies (McIntosh et al. 2003). We also included one accession from Afghanistan (IL 369) that has a dominant *Vrn-A1*

Table 1 Wheat varieties with known *Vrn-1* alleles based on previously published genetic studies

Variety	PI	Proposed allele combination[a]	Promoter *Vrn-A1*[b]	Intron 1 deletion[c]
Triple Dirk C		*vrn-A1 vrn-B1 vrn-D1*	Winter	None
Triple Dirk B		*vrn-A1* ***Vrn-B1*** *vrn-D1*	Winter	B
Bersee	PI 168661	*vrn-A1* ***Vrn-B1*** *vrn-D1*	Winter	B
Festiguay	PI 330957	*vrn-A1* ***Vrn-B1*** *vrn-D1*	Winter	B
Mara	PI 244854	*vrn-A1* ***Vrn-B1*** *vrn-D1*	Winter	B
Mil'turum	PI 155125	*vrn-A1* ***Vrn-B1*** *vrn-D1*	Winter	B
Noe	PI 193158	*vrn-A1* ***Vrn-B1*** *vrn-D1*	Winter	B
Spica	PI 213830	*vrn-A1* ***Vrn-B1*** *vrn-D1*	Winter	B
Shortadinka	PI 326303	***Vrn-A1 Vrn-B1*** *vrn-D1*	A insertion	B
Takary	PI 483058	***Vrn-A1 Vrn-B1*** *vrn-D1*	A insertion	B
Triple Dirk D		***Vrn-A1*** *vrn-B1 vrn-D1*	A insertion	None
Cadet	CItr 12053	***Vrn-A1*** *vrn-B1 vrn-D1*	A insertion	None
Conley	CItr 13157	***Vrn-A1*** *vrn-B1 vrn-D1*	A insertion	None
Diamant II	PI 190489	***Vrn-A1*** *vrn-B1 vrn-D1*	A insertion	None
Reward	PI 351819	***Vrn-A1*** *vrn-B1 vrn-D1*	A insertion	None
Saitama 27	PI 155279	***Vrn-A1*** *vrn-B1 vrn-D1*	A insertion	None
Afghanistan LR[b]	IL 369	***Vrn-A1*** *vrn-B1* ***Vrn-D1***	Winter	A and D
Norin 61	PI 235236	*vrn-A1 vrn-B1* ***Vrn-D1***	Winter	D
Shinchunaga	PI 197128	*vrn-A1 vrn-B1* ***Vrn-D1***	Winter	D
Shirasagi Komugi	PI 360870	*vrn-A1 vrn-B1* ***Vrn-D1***	Winter	D
Ushio Komugi	PI 384010	*vrn-A1 vrn-B1* ***Vrn-D1***	Winter	D
Triple Dirk E		*vrn-A1 vrn-B1* ***Vrn-D1***	Winter	D
Chinese Spring		*vrn-A1 vrn-B1* ***Vrn-D1***	Winter	D
Langdon[d]		***Vrn-A1*** *vrn-B1*	Winter	A

[a]The data are compiled in McIntosh et al. (2003). Dominant spring alleles are indicated in bold

[b]From Yan et al. 2004a

[c]The last column indicates the genomes showing deletions in intron 1 as determined in this study

[d]This study

allele with an identical promoter region to the recessive *vrn-A1* allele (Yan et al. 2004a), 24 spring durum wheat varieties, 37 winter varieties of common wheat and 117 spring varieties of common wheat that were grown in California and Argentina (Table 2). Nulli-tetrasomic lines of *T. aestivum* cv. Chinese Spring, N5AT5D, N5BT5D and N5DT5B (Sears 1966) were used to validate the genome specificity of the designed primers.

Three F_2 populations obtained from crosses between spring and winter accessions were used to test the linkage between spring growth habit and *VRN-A1*, *VRN-B1* and *VRN-H1* deletions in intron 1. For the *VRN-A1* locus we used a population generated from a cross between Langdon and the winter durum wheat Durelle. For the *VRN-B1* locus we used the hexaploid F_2 population from the cross Triple Dirk B × Triple Dirk C (Barrett et al. 2002), kindly provided by Dr. Kim Kidwell. For the *VRN-H1* locus we reanalyzed a population derived from a cross between the spring barley variety Morex (*Vrn-H1vrn-H2*) and the winter wild barley *H. spontaneum* koch (OSU6, PBI004-7-0-015) (Chen 2002).

Barley *VRN-H1* orthologues were sequenced from the varieties Dicktoo, Morex, Strider and Oregon Wolf Barley Dominant (OWB-D). Dicktoo is a vernalization-insensitive, late flowering genotype, while Morex and OWB-D are spring genotypes (Wolfe and Franckowiak 1991; Hayes et al. 1997). Strider is a vernalization-responsive winter barley genotype (Karsai et al. 2004).

Cloning and sequencing

Genomic DNA from leaves of a single plant was isolated as described before (Dubcovsky et al. 1994). PCRs were performed using Taq DNA Polymerase (Promega, Madison, WI, USA) and the FailSafe PCR System with PreMix E (Epicentre, Madison, WI, USA). PCR prod-

Table 2 Survey of wheat varieties grown in Argentina and California (1930–2004) with previously unknown vernalization genes

Allele combination[a]	Wheat varieties grown in Argentina and California
Winter (6x)	
vrn-A1 vrn-B1 vrn-D1	Argentina: General Roca, Klein 32, Klein Rendidor, Prointa Puntal, Prointa Super US: Andrews, Barbee, Bonne Ville, Eltan, Garland, Gene, Hill 81, Hyak, Kmor, Lambert, Mac Vicar, Madsen, Malcolm, Meridian, Newton, Nugaines, Phoenix, Promontory, Red Chief, Rod, Rohde, Rulo, Stephens, Tascosa, Triumph 64, Turkey, Yamhill, Wincora Others: Ranyaya, Bezostaya, VPM1, Mercia
Spring (4x)	
Vrn-A1c *vrn-B1*	Argentina: Bonaerense INTA Cumenay, Bonaerense INTA Facón, Bonaerense Quilacó, Buck Ambar, Buck Esmeralda, Buck Topacio US: Aldura, Altar 84, Aruba, Carcomun's, Duraking, Durex, Durfort, Eddie, Imperial, Leeds, Mexicali 75, Minos, Ocotillo, Reva, Ria, Westbred 881, Westbred Laker, Westbred Turbo
Spring (6x)	
Vrn-A1a/b *vrn-B1 vrn-D1*	Argentina: Buck Biguá, Buck Catriel, Buck Guaraní, Buck Palenque, Cooperación Millán, Diamante INTA, Klein Flecha, Leones INTA, Pampa INTA, Prointa Bonaerense Redomón, Prointa Elite, Prointa Molinero US: Anza, Big Club 60, Blanca, Brooks, Calorwa, Canthatch, Centennial, Challenger, Copper, Dirkwin, Klasic, Marshall, Poco Red, Rich, Spillman, Westbred 926
Vrn-A1a/b Vrn-B1 *vrn-D1*	Argentina: Bonaerense Pasuco, Buck Arriero, Buck Brasil, Buck Guatimozín, Buck Manantial, Buck Ñandú, Cooperación Liquén, Granero INTA, Klein Chajá, Prointa Bonaerense Alazán, Prointa Granar US: Len, Owens, Probrand 755, Siete Cerros 66 (CIMMYT), Stoa, Sunstar II, Sunstar Promise, Treasure, Twin
Vrn-A1a/b *vrn-B1* ***Vrn-D1***	Argentina: ACA 601, Buck Chambergo, Buck Namuncurá, Buck Patacón, Buck Yapeyú, Las Rosas INTA, Prointa Imperial US: Serra, Sonora 64 (CIMMYT), Waduel 94.
Vrn-A1a/bVrn-B1Vrn-D1	Argentina: ACA 302, Buck Mataco, Buck Pingo, INIA Tijereta, Prointa Colibrí. US: Pomerelle, Wawawai, Yaqui 54 (CIMMYT).
vrn-A1 Vrn-B1 ***Vrn-D1***	Argentina: Bonaerense INTA Potrillo, Buck Charrúa, Buck Guapo, Buck Pampero, Buck Quequén, Buck Sureño, Buck Yatasto, INIA Churrinche, Klein Don Enrique, Klein Petiso, Klein Sagitario, Klein Salado, Prointa Oasis. US: Inia 66R, Penawawa, Pitic 62 (CIMMYT), Poso 48, Ramona 50, RSI 5, Whitebird.
vrn-A1 vrn-B1 Vrn-D1	Argentina: Marcos Juárez INTA, Prointa Gaucho US: Cajeme 71, Express, Kern, Probred, UC1037, UC1041, UC1107, UC1358, Westbred 911
vrn-A1 ***Vrn-B1 Vrn-D1***	Argentina: ACA 303, Bonaerense INTA Payador, Bordenave Puan SAG, Buck Farol, Buck Poncho, Klein Escorpión, Klein Escudo, Klein Estrella, Klein Jabalí, Klein Martillo, Klein Pegaso, Klein Proteo. US: Alpowa, Clear White, Nainari 60 (CIMMYT), Shasta, Tadinia, Tammy, UC1128, Vanna

[a] Information for the *Vrn-1* promoter regions is from Yan et al. (2004). *Vrn-A1a* and *Vrn-A1b* indicate promoter insertions or deletions, respectively (no intron deletion); *Vrn-A1c*, intron 1 deletion in the A genome copy; *Vrn-B1*, intron 1 deletion in the B genome copy; *Vrn-D1*, intron 1 deletion in the D genome copy. *vrn-A1, vrn-B1* and *vrn-D1* indicate no indels in the intron or promoter region compared to the winter allele

ucts were isolated from agarose gels, cloned into pGEM-T Easy (Promega), and sequenced in both directions with T7 and SP6 primers; sequencing was completed by primer walking.

Conserved primers were designed using the PRIMER3 program (Rozen and Skaletsky 2000). Genome-specific primers were initially designed by hand to include regions polymorphic among genomes, and then checked using the Oligonucleotide Properties Calculator (Version 3.07, Northwestern University, Chicago, IL). Sequences from overlapping PCR products were assembled using the Vector NTI program (version 9.0.0; InforMax, Frederick, Md.).

The complete genomic sequence of the *T. monococcum VRN-A*^{m}1 gene (Yan et al. 2003), the promoter sequences from the genes *VRN-A1, VRN-B1* and *VRN-D1* (Yan et al. 2004a), and the cDNA sequences of the *VRN-B1* (Danyluk et al. 2003), *VRN-D1* (Murai et al. 1998) and *VRN-H1* genes (Schmitz et al. 2000) served as the starting points for the PCR-based cloning of the different *VRN-1* alleles. We first obtained complete genomic sequences of *VRN-1* including approximately 500 bp of the 5′ and 300 bp of the 3′ regions from BAC clones using overlapping PCR fragments. We sequenced this gene from BACs 631P8 (*VRN-H1*, AY750995) from the barley variety Morex (Yu et al. 2000), 1256C17 (*VRN-A1*, AY747598) and 1225D16 (*VRN-B1*, AY747602) from the tetraploid wheat variety Langdon (Cenci et al. 2003), and 22J2 (*VRN-D1*, AY747605) from an *Eco*RI BAC library of *T. tauschii* (Xu et al. 2002).

We then used the sequence information from the wheat BAC clones to develop genome-specific primers with which to clone the individual genes from the polyploid wheat species. For wheat, complete sequences were obtained for the *VRN-A1* genes from TDC (AY747600), TDD (AY747601) and IL 369 (AY747599); for the *VRN-B1* genes from TDC (AY747604) and TDB (AY747603); and for the *VRN-D1* genes from TDC (AY747606) and TDE (AY747597). For barley, an additional complete sequence was obtained for the recessive *vrn-H1* allele from Strider (AY750993). The *VRN-H1* intron 1 region was also cloned and sequenced from Dicktoo (AY750994) and Oregon Wolf Barley Dominant (AY750996). All polymorphisms among alleles from the same genome were confirmed by cloning and sequencing amplification products from at least two independent PCRs.

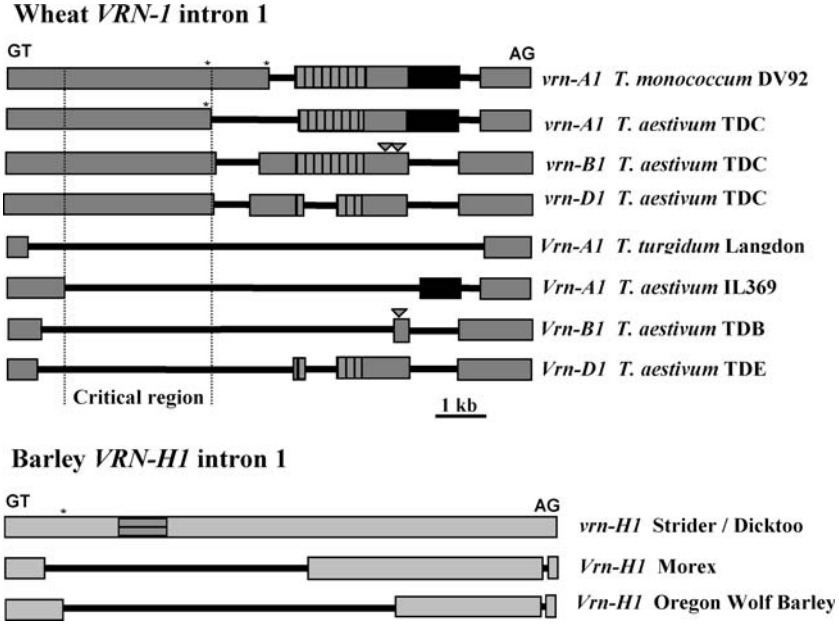

Fig. 1 Schematic representation of variation in intron 1 of *VRN-1* among genomes, and between dominant and recessive alleles in wheat and barley. Deletions of less than 100 bp are not represented. The *asterisks* represent MITEs. In wheat, the *inverted triangles* represent insertions of AU SINE elements in the B genome. The *black rectangle* represents a part of a *Sukkula* retroelement present only in the A genome. The *gray rectangle with vertical stripes* represents a region with significant similarity to a polyprotein gene in *Solanum demissum* (AAT40504.1, 54% similar), and to a repetitive element inserted in intron 10 of the Starch Synthetase I gene in *T. tauschii* (AF091802, 86% identical) and intron 2 of the barley *Hotr1* gene (AJ001317.1). In barley the *rectangle with the horizontal line* represents a retrotransposon similar to *Lolaog* (AY268139)

Results

Allelic variation at *VRN-A1*

Among the A genome sequences, the sequence of *VRN-A1* from *T. monococcum* (13,465 bp) showed the greatest divergence (98% identity) when compared with the *VRN-A1* sequences from the polyploid species, showing 227 unique SNPs and 27 small indels (1–14 bp). In addition, one large deletion (1375 bp) was observed in *VRN-A1* intron 1 in the four polyploid accessions relative to *T. monococcum* (Fig. 1). The 1375-bp deletion in the A

genome of the polyploid species was replaced by a 120-bp sequence with no significant similarity to the deleted region. The 120-bp sequence included a perfect 45-bp duplication of a region upstream of the 3′ border of the deletion and a 32-bp duplication that was 94% identical to a region downstream of the 5′ border of the deletion.

The four A genome sequences from the polyploid species showed limited sequence variation. The dominant *Vrn-A1* allele from hexaploid land race IL 369 (Afghanistan) was the most divergent, with eight unique SNPs, five unique 1-bp indels and one large 5504-bp deletion in intron 1 relative to the recessive *vrn-A1* allele in TDC (1169–6672 bp; Fig. 1). Among the unique SNPs and 1-bp indels, one was found in the promoter, eight in the first intron, and one each in introns 2, 4 and 6. The last SNP was found in exon 7 and resulted in a conservative amino acid polymorphism (Ala/Val).

The dominant *Vrn-A1* allele from TDD was very similar to the recessive *vrn-A1* alleles from TDC. They have four mutations in common that differentiate them from the *Vrn-A1* alleles from Langdon and IL 369. The major difference between the TDD and TDC alleles was the insertion of the *Spring* foldback element in the promoter region of the dominant *Vrn-A1* allele of TDD (Dubcovsky and Yan 2003; Yan et al. 2004a). In addition, these two sequences differ in one SNP in intron 1 and three SNPs in intron 2. The last two SNPs from intron 2 lie within a 229-bp region that is deleted in all dominant and recessive *VRN-B1* and *VRN-D1* alleles. Similarly, the intron 1 SNP was within the retrotransposon *Sukkula*, which was absent in all the B and D genome alleles (Fig. 1). The "T" allele for the first SNP in intron 2 was found only in the recessive TDC allele, whereas the "C" allele was present in all the other dominant and recessive *VRN-1* alleles from the A, B and D genomes. Therefore, none of the four SNPs between TDD and TDC *VRN-A1* alleles was consistently associated with dominant and recessive alleles in other accessions. In contrast, the foldback element insertion in the promoter region was previously associated with a dominant *Vrn-A1* allele in a large germplasm collection (Yan et al. 2004a).

The dominant *Vrn-A1* allele from the tetraploid variety Langdon showed a 7222-bp deletion in intron 1 (391–7612 bp, Fig. 1) relative to the TDC *vrn-A1* allele. This 7222-bp deletion was replaced by a 52-bp fragment that showed no significant similarity to the deleted region. This 52-bp fragment included a 36-bp segment that was 96% identical to a region 54 bp upstream from the start of the deletion, and a 16-bp segment 100% identical to a region 114 bp upstream from the start of the deletion.

We confirmed that the 7222-bp deletion co-segregated with the spring growth habit in an F_2 population from the cross between the tetraploid varieties Langdon (intron deletion, spring) and Durelle (no intron deletion, winter). Among the 80 F_2 plants analyzed, 18 showed a winter growth habit, indicating segregation for a single dominant gene (3:1 segregation test $\chi^2 = 0.3$, $P = 0.61$). Analysis of the 18 winter plants with the two sets of primers for this deletion (see below) demonstrated that all winter plants were homozygous for the absence of the deletion. At least one copy of the allele with the intron 1 deletion was detected in all the spring lines analyzed. These results indicate that Langdon has a dominant *Vrn-A1* allele and a recessive *vrn-B1* allele.

Allelic variation at *VRN-B1*

The 13,465-bp region in *T. monococcum VRN-A*^{m}*1* corresponded to a 13,141-bp region in the recessive *vrn-B1* alleles from TDC and Langdon. These two recessive *vrn-B1* alleles revealed five SNPs and two 1-bp indels in the first two introns. Since both alleles are recessive, we concluded that these mutations were not essential for the determination of the winter growth habit.

The dominant *Vrn-B1* allele from TDB differed from both recessive alleles by a 6850-bp deletion in intron 1 (from 652 to 7501 bp, counting from the start of intron 1 in TDC). In addition to this large deletion, only two SNPs in introns 1 and 2 differentiate the sequences from TDC and TDB. However, these two SNPs in TDB *Vrn-B1* alleles were identical to the recessive *vrn-B1* allele from Langdon, indicating that they are not relevant for the determination of spring growth habit.

The 6850-bp deletion co-segregated with the spring growth habit in an F_2 population derived from the cross between TDB and TDC. This population was previously characterized for growth habit and for markers linked to the segregating *VRN-B1* gene (Barrett et al. 2002). In this study we found that the 28 winter lines were homozygous for the absence of the 6850-bp deletion, whereas the 62 spring lines were homozygous or heterozygous for the presence of this deletion.

Allelic variation at *VRN-D1*

The total lengths of the sequences of the recessive *vrn-D1* alleles from the winter lines TDC and *T. tauschii* were 12,335 and 12,224 bp, respectively. However, the dominant *Vrn-D1* allele from spring TDE was only 8100 bp long. The smaller size of the TDE allele was due to a 4235-bp deletion in intron 1 (625–4859 bp, counting from the start of the intron 1 in TDC), which is flanked by TCCG direct repeats. Besides this large deletion, no other differences were found in the 8100-bp compared between the dominant *Vrn-D1* from TDC and the recessive *vrn-D1* allele from TDE.

Sequence comparison between the *VRN-D1* alleles from TDC and *T. tauschii* revealed ten SNPs, two 1-bp and one 109-bp indels in the first two introns, and one SNP in the third exon. The mutation in the third exon did not result in an amino acid exchange.

Allelic variation at *VRN-H1*

Comparisons between the two complete barley sequences corresponding to the dominant *Vrn-H1* allele

from Morex and the recessive *vrn-H1* allele from Strider showed identical exon sequences and good conservation in the promoter region (2 kb, 99.2% identical) and intron regions (99.5% identical). The first 450 bp upstream of the start codon differed only in two SNPs and one 4-bp indel in the TCGC repeated region of the 5′UTR. The major difference was a 5.2-kb deletion in the dominant *Vrn-H1* allele relative to the recessive *vrn-H1* allele (798–5954 bp from the start of intron 1 in Strider; Fig. 1). One border outside the deletion and the other one inside include the same ACC sequence, suggesting that the deletion might have originated by slippage replication (Ramakrishna et al. 2002).

The *VRN-H1* intron 1 sequences from Dicktoo and Strider were 100% identical, but the OWB-D intron 1 showed a 6.4-kb deletion (1146–7559 bp from the start of intron 1). The 6.4-kb deletion was replaced by a 31-bp fragment delimited by inverted GCC repeats, and including a 21-bp region identical to a region close to the 5′ border of the deletion that includes the end of a MITE. The different start and endpoints of this deletion relative to the one observed in Morex suggest that these two deletions arose independently. Morex and OWB-D share a 42-bp deletion in the 3′ region of intron 1 that is absent in Strider (Fig. 1).

The 5.2-kb deletion from Morex co-segregated with the spring growth habit in the F_2 population (91 plants) from the cross between Morex and *H. spontaneum* (Chen 2002). Since this population was also segregating for *VRN-H2* it was possible to study the epistatic interactions between the *Vrn-H1* allele with the intron 1 deletion and *VRN-H2*. A two-way factorial analysis of variance showed a significant interaction between *VRN-H1* and *VRN-H2* ($P = 0.01$). Therefore, we analyzed the effect of each locus within each class of the other locus by four one-way analyses of variance.

We first analyzed the effect of the *VRN-H1* intron 1 deletion within the two *VRN-H2* classes. Among the plants carrying the recessive *vrn-H2* allele no significant effect on flowering time was observed between the plants with and without the *VRN-H1* intron 1 deletion ($P = 0.69$). However, among the plants carrying the dominant *Vrn-H2* allele, plants carrying at least one copy of the *VRN-H1* intron 1 deletion flowered 35 days earlier than the plants without the deletion ($P < 0.0001$).

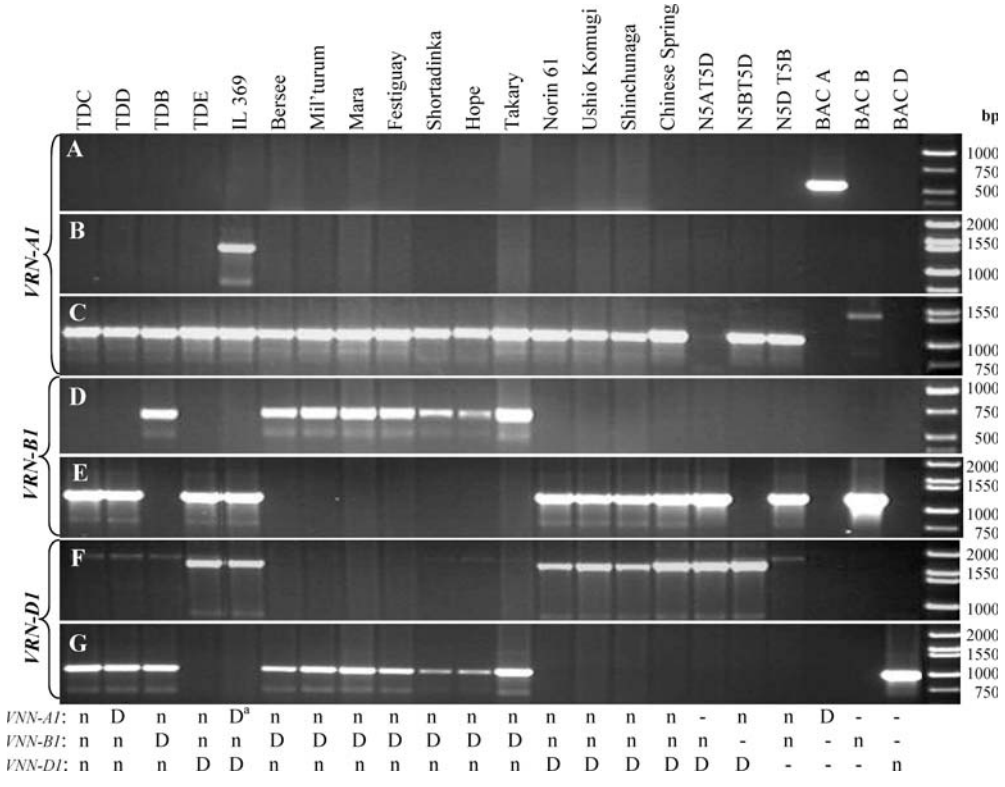

Fig. 2 a–g PCR amplification using genome-specific primers to detect the presence and the absence of deletions in the first intron of the *VRN-1* gene in polyploid wheat. BAC A: Langdon BAC 1256C17 including *VRN-A1*, BAC B: Langdon BAC 1225D16 including *VRN-B1*, BAC D: *T. tauschii* BAC 22J2 including *VRN-D1*. The products shown were obtained with primers designed to detect deletion in *VRN-A1* from tetraploid wheat (**a**), deletion in *VRN-A1* from hexaploid wheat (**b**), absence of deletion in *VRN-A1* (a faint and larger non-specific band was observed in BAC B) (**c**),deletion in *VRN-B1* (**d**), absence of deletion in *VRN-B1* (**e**), deletion in *VRN-D1* (a faint and slightly larger non-specific band was observed in some accessions without the deletion) (**f**), absence of deletion in *VRN-D1* (**g**). The allelic constitution of each accession is indicated in Table 1 and summarized below *panels* A-G. *n*, allele with no deletion in intron 1; –, complete gene absent; *D*, dominant allele with intron 1 deletion. TDD, Shortadinka and Takary have the dominant *Vrn-A1* allele with the promoter insertion. D^a, IL 369 showed both presence and absence of the intron 1 deletion suggesting a duplication of this region

Similar results were obtained when we analyzed the effect of the *VRN-H2* gene deletion within the two *VRN-H1* classes. No significant effects for the *Vrn-H2* alleles were observed among plants carrying at least one *Vrn-H1* allele with the intron deletion ($P = 0.54$). However, among the plants with the recessive *vrn-H1* allele, those carrying the recessive *vrn-H2* allele flowered 34 days earlier than those with the dominant *Vrn-H2* allele ($P = 0.0003$).

Comparisons among different genomes

The comparison between the DNA sequences of the recessive *vrn-1* alleles from Strider barley and diploid wheat showed 97% identity in the exon regions, 89% identity in the first 2 kb of the promoter region, and 88% identity in introns. A region of relatively high sequence identity (92%) between diploid wheat and barley was identified in intron 1 between positions 1251 and 1687 bp (counting from the start of Strider *vrn-H1* intron 1). This 440-bp region (436 bp in barley) starts at the point where all the large deletions in intron 1 in dominant alleles overlap (border of the IL 369 deletion; ''Critical region'' in Fig. 1) and is flanked by 1.2-kb and 2.4-kb regions with lower sequence identity (87%) between these two genera. The 2.4-kb region excludes a unique 1051-bp segment in barley similar to the retrotransposon Lolaog_184G9-1 (AY268139) and flanked by ACCTAT direct repeats (Fig. 1).

No significant similarity was detected between the intron 1 regions located between 5.1 and 10.5 kb in Strider and 4 and 9.5 kb in *T. monococcum* (counting from the start of intron 1). These two non-orthologous regions include pieces of a *Sukkula* retroelement and inverted and direct repeats, and show similarities to putative repetitive genomic regions in *T. tauschii* and rice (Fig. 1). These results suggest that this 5.5-kb region of intron 1 is not important for determination of the winter growth habit by these two recessive alleles.

Comparison of the wheat and barley alleles carrying deletions in the first intron showed that they all overlap in a 4.2-kb region. The left border of this region is defined by the start of the intron 1 deletion in IL 369 (1.2 kb), which is the deletion with the most distal startpoint. The right border of the overlapping region is defined by the end of the *VRN-D1* deletion in TDE (5.4-kb from the start of intron one in *T. monococcum*), which has the most proximal end of the six intron 1 deletions (Fig. 1; ''Critical region'').

The right border of the ''critical region'' can be further refined by comparing the different recessive alleles and assuming that a region that is relevant for the vernalization requirement should be conserved among the different genomes. The presence of a large deletion starting 4.1 kb downstream from the start of intron 1 in the recessive *vrn-A1* allele defined a more proximal right border for the conserved region. This region seems relatively prone to deletions because it also included a 720-bp deletion in the B and D genomes relative to *T. monococcum* (flanked by ATTT direct repeats) and a 560-bp deletion in *T. monococcum* relative to the B and D genomes (flanked by TGCCTACAC direct repeats, Fig. 1). A comparison of the intron 1 sequences between wheat and barley showed that sequence similarity was interrupted 4.0 kb downstream from the start of intron 1. The 2.8-kb region between 1.2 and 4.0 kb showed good conservation among the three wheat genomes and also between wheat and barley.

Table 3 PCR markers for the different *VRN-1* alleles in wheat and barley

PCR marker	Primers		Expected size of product (bp)	Annealing temperature (°C)[a]
	Name	Sequence (5′ to 3′)		
Langdon *Vrn-A1* deletion	Ex1/C/F	GTTCTCCACCGAGTCATGGT	522	55.6
	Intr1/A/R3	AAGTAAGACAACACGAATGTGAGA		
IL 369 *Vrn-A1* deletion	Intr1/A/F2	AGCCTCCACGGTTTGAAAGTAA	1170	58.9
	Intr1/A/R3	AAGTAAGACAACACGAATGTGAGA		
Vrn-A1 Non-deletion	Intr1/C/F	GCACTCCTAACCCACTAACC	1068	56.0
	Intr1/AB/R	TCATCCATCATCAAGGCAAA		
TDB *Vrn-B1* deletion	Intr1/B/F	CAAGTGGAACGGTTAGGACA	709	58.0
	Intr1/B/R3	CTCATGCCAAAAATTGAAGATGA		
Vrn-B1 Non-deletion	Intr1/B/F	CAAGTGGAACGGTTAGGACA	1149	56.4
	Intr1/B/R4	CAAATGAAAAGGAATGAGAGCA		
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		
Morex *Vrn-H1* deletion	Intr1/H/F1	GCTCCAGCTGATGAAACTCC	474	55.0
	Intr1/H/R1	CTTCATGGTTTTGCAAGCTCC		
Vrn-H1 Non-deletion	Intr1/H/F3	TTCATCATGGATCGCCAGTA	403	55.0
	Intr1/H/R3	AAAGCTCCTGCCAACTACGA		

[a]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

PCR primers for the deletions in intron 1 of *VRN-1* in wheat

To facilitate the screening of large wheat germplasm collections for *VRN-1* intron 1 deletions, we developed two pairs of genome-specific primers for each deletion. The first primer pair was designed to produce an amplification product only when the deletion was present, whereas the second primer pair was designed to produce an amplification product when the deletion was absent. These two primer pairs can be combined to generate a codominant marker.

The genome-specificity of the different primers was confirmed by using DNAs from the three different BACs and genomic DNAs from nulli-tetrasomic lines of Chinese Spring N5AT5D, N5BT5D and N5DT5B (Fig. 2).

Three PCR markers were developed for the A genome alleles (Table 3). The primer pair Ex1/C/F and Intr1/A/R3 was used to detect the deletion present in Langdon (Fig. 2a), whereas the primer pair Intr1/A/F2 and Intr1/A/R3 was used to detect the *VRN-A1* intron 1 deletion found in IL 369 (Fig. 2b). The third pair of primers, Intr1/C/F and Intr1/AB/R, was used as a positive amplification control for the absence of these two deletions (Fig. 2c).

Surprisingly, the fragment indicating the absence of the intron 1 deletion was detected in IL 369 in addition to the fragment indicating the presence of the deletion (Fig. 2b, c). Ten individual IL 369 plants showed the same pattern, suggesting that this amplification was more likely to be the result of a duplication of this region than of heterozygosity for the intron 1 deletion. This duplication does not seem to include the complete *VRN-A1* gene because Southern hybridization of IL 369 genomic DNA with a probe including *VRN-A1* exons 4–8 did not reveal additional restriction fragments. Further studies will be necessary to explain the results from this line.

The B genome-specific primer pair Intr1/B/F and Intr1/B/R3 (Table 3) was designed to test for the presence of the large deletion in the dominant *Vrn-B1* allele. When the deletion was present these primers generated a 709-bp amplification product (Fig. 2d). They amplified a 709-bp fragment in TDB and all the other eight varieties known to carry the dominant *Vrn-B1* allele (Table 1, Fig. 2d), suggesting that these accessions all have the same deletion. All these varieties with the *Vrn-B1* allele showed no amplification product with primers Intr1/B/F and Intr1/B/R4 (Table 3, Fig. 2e). This pair of primers was designed as a positive control for the absence of the deletion. Varieties with the recessive *vrn-B1* allele yielded a 1149-bp PCR product with these primers (Table 3, Fig. 2e).

The D genome-specific primer pair Intr1/D/F and Intr1/D/R3 amplified a 1671-bp band only when the dominant allele *Vrn-D1* with the large intron deletion was present (Fig. 2f). In contrast, primers Intr1/D/F and Intr1/D/R4 produced a 997-bp product only when the deletion was absent (*vrn-D1* allele, Fig. 2g). TDE and

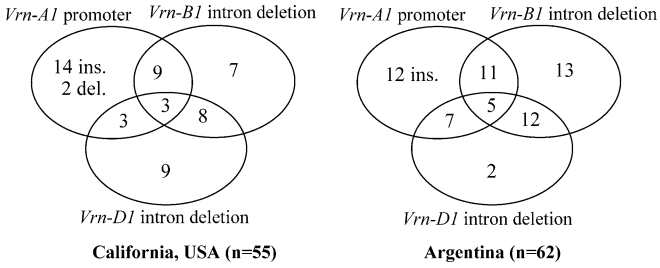

Fig. 3 Distribution of different *VRN-1* allelic combinations in hexaploid spring wheat varieties grown in Argentina and California

the other six varieties with known dominant *Vrn-D1* alleles (Table 1, Fig. 2f) yielded PCR fragments of similar size, suggesting that all have the same deletion. This result was expected, because the dominant *Vrn-D1* allele was introgressed into the Italian variety Mentana from the Japanese variety Akakomugi, and then transferred from Mentana into the Mexican semi-dwarf cultivars Lerma Rojo and Sonora 64, initiating a worldwide distribution of this allele (Stelmakh 1998).

Primer pair Intr1/H/F1 and Intr1/H/R1 (Table 3) amplified a 474-bp band when the Morex *Vrn-H1* allele was present. Primers Intr1/H/F3 and Intr1/H/R3 (Table 3) produced a 403-bp product only in the absence of these deletions (*vrn-H1* allele).

VRN-1 allele combinations in wheat varieties grown in Argentina and California

No deletions in the first intron were observed in the *VRN-1* loci of the A, B and D genomes in the 37 winter varieties of common wheat analyzed in this study (Table 2). Conversely, all the 51 spring accessions that were previously found to have a *VRN-A1* promoter identical to that associated with the recessive *vrn-A1* allele (Yan et al. 2004a) showed deletions in the first intron of the *VRN-B1* gene (20 varieties), the *VRN-D1* gene (11 varieties) or both (20 varieties) (Table 2, Fig. 3). None of the common wheat varieties grown in Argentina or California showed *VRN-A1* intron 1 deletions similar to those found in tetraploid wheat or IL 369. The *VRN-B1* and *VRN-D1* intron 1 deletions, combined with the *VRN-A1* promoter insertions and deletions described before (Yan et al. 2004a), were sufficient to explain the spring growth habit of the 117 common wheat spring varieties analyzed in this study (Table 2, Fig. 3).

All 24 accessions of tetraploid varieties grown in Argentina and California showed a deletion in the *VRN-A1* intron 1, which was of similar size to that found in the tetraploid Langdon. The *Vrn-B1* deletion was not detected in these varieties. As expected, the winter tetraploid variety Durelle showed no deletions in intron 1 of the *VRN-A1* or the *VRN-B1* gene.

The frequencies of each of the dominant alleles in Argentina (Table 4) were very similar to those previously reported for a set of 101 varieties from Latin

Table 4 Frequencies of the three dominant alleles among the spring wheat varieties grown in Argentina and California (1930–2004) and comparison of the frequencies of the observed allele combinations with those predicted based on the individual allele frequencies

Allele frequency[a]	**Argentina**	**Latin America**[b]	**California**	**USA and Canada**[b]	**Overall**
Vrn-A1	56.5	56.4	56.4	91.1	80.8
Vrn-B1	66.1	61.4	49.1	60.0	62.4
Vrn-D1	41.9	43.6	41.8	6.7	19.6
No. of varieties tested	62	101	55	45	647
Allele combination in spring varieties	**Observed Argentina**	**Expected Argentina**[c]	**Observed California**	**Expected California**[c]	**Observed overall**[b]
Vrn-A1 Vrn-B1 Vrn-D1	8.1	17.1	5.5	13.3	0.6
Vrn-A1 Vrn-B1 *vrn-D1*	17.7	23.7	16.4	18.5	48.4
Vrn-A1 *vrn-B1* ***Vrn-D1***	11.3	8.8	5.5	13.8	7.3
Vrn-A1 *vrn-B1 vrn-D1*	19.4	12.1	29.1	19.2	24.6
vrn-A1 ***Vrn-B1 Vrn-D1***	19.4	13.2	14.5	10.3	6.0
vrn-A1 ***Vrn-B1*** *vrn-D1*	21.0	18.3	12.7	14.3	7.4
vrn-A1 vrn-B1 ***Vrn-D1***	3.2	6.8	16.4	10.7	5.7

[a]The top three rows show the individual allele frequencies, the last seven rows list the frequencies of the indicated allele combinations. Values are given as percentages of the total

[b]Data from Stelmakh (1998)

[c]Expected numbers of different allele combinations based on frequencies of the different alleles calculated in the first three rows. The winter combination *vrn-A1 vrn-B1 vrn-D1* was then eliminated, and the percentages were adjusted to add to 100% (*VRN-A1* allele frequency × *VRN-B1* allele frequency × *VRN-D1* allele frequency)/(1-(*vrn-A1* × *vrn-B1* × *vrn-D1*))

America (Stelmakh 1998). However, the frequencies of the dominant alleles in California (Table 4) were more similar to those in Argentina than to the ones reported for a collection of 45 varieties from Canada and the USA, or for a large worldwide collection (Stelmakh 1998).

The frequencies of the different *VRN-1* allele combinations in Argentina and California were similar to each other (Table 4, χ^2 test $P = 0.24$) but very different (χ^2, $P < 0.0001$) from the ones reported for a large worldwide collection previously characterized for *VRN-1* allele combinations (Stelmakh 1998).

Discussion

Intronic regulatory elements

Introns are non-coding sequences located between the coding exons, and are removed by splicing during RNA processing. In recent years, intronic regulatory elements (IREs; Yutzey et al. 1989) have been identified which function as enhancers, repressors and promoters of gene transcription (Busch et al. 1999; Deyholos and Sieburth 2000; Kapranov et al. 2001; Fiume et al. 2004). In many cases, IREs reside within the first intron (Wang et al. 2002; Gazzani et al. 2003; Michaels et al. 2003; Fiume et al. 2004).

An example of an IRE that is relevant for our discussion is provided by the *Arabidopsis FLOWERING LOCUS C* (*FLC*) gene (Sheldon et al. 2002; Gazzani et al. 2003; Michaels et al. 2003). This gene encodes a MADS-box transcription factor that plays a central role in the vernalization pathway in *Arabidopsis* (Michaels and Amasino 1999; Sheldon et al. 1999). *FLC* suppresses flowering and is permanently down regulated by cold treatment. It was recently demonstrated that vernalization requires epigenetic silencing of *FLC* by histone methylation and acetylation in specific regions of intron 1 (He et al. 2003; Bastow et al. 2004). This chromatin remodeling in intron 1 results in permanent suppression of *FLC*, which allows the expression of the genes *SOC1, LFY* and *AP1*, thus inducing flowering (Jack 2004).

The *VRN-1* gene in Triticeae is not orthologous to the *Arabidopsis FLC* gene, but in both exon 1 encodes a MADS-box domain and is followed by a large intron 1. Several indirect lines of evidence support the hypothesis that in *VRN-1* important regulatory regions reside within the large intron 1. First, six independent deletions in the first intron of *VRN-1* were found in wheat and barley accessions carrying dominant *Vrn-1* alleles. Second, all six deletions overlap in a 2.8-kb region, which was well conserved among the recessive *vrn-1* alleles in the different genomes. Third, those accessions with deletions in the first intron showed no (e.g. *VRN-D1*) or very few SNPs relative to their respective recessive alleles in the rest of the gene. Furthermore, these few SNPs were not consistently associated with different dominant and recessive alleles, suggesting that they were not critical for the determination of growth habit. Fourth, deletions in the first intron were perfectly correlated with the spring growth habit in the three mapping populations analyzed in this study, and in all the 24 tetraploid and 51 hexaploid wheat varieties previously shown to have identical promoter sequences in dominant and recessive alleles (Yan et al. 2004a).

Finally, the intron 1 deletions provide a simple explanation for the observed dominance and epistatic interactions between vernalization genes. The elimination of a repressor recognition site (e.g. a direct or indirect target of VRN-H2) from the first intron provides a simple explanation for the dominant nature of the *Vrn-A1, Vrn-B1, Vrn-D1* and *Vrn-H1* alleles. The presence of a single copy of the meristem identity gene

lacking this recognition site would be sufficient to induce flowering without vernalization.

Two *VRN-1* regulatory sites affect the vernalization response

Mutations in the first intron of *VRN-1* are not the only ones associated with dominant *Vrn-1* alleles. Different deletions in the promoter of the *VRN-A*^{m}*1*gene have been correlated with dominant alleles for spring growth habit in diploid wheat (Yan et al. 2003). In addition, an insertion in the promoter region of the *VRN-A1* gene from TDD was associated with the dominant *Vrn-A1* allele in a large germplasm collection of common wheat (Yan et al. 2004a). Except for the promoter insertion, the dominant *Vrn-A1* allele from TDD is almost identical to the recessive *vrn-A1* allele from TDC. The four SNPs detected between these two alleles were not correlated with dominant or recessive alleles in other *VRN-1* genes. This result suggests that the promoter mutation in TDD *VRN-A1* might be sufficient for the determination of spring growth habit.

In addition, all the accessions with large deletions in the first intron of *VRN-1* have promoter regions that are identical to those of their corresponding recessive alleles, suggesting that the intron 1 mutations might also be sufficient for the determination of the spring growth habit. Based on the previous observations, we hypothesize that both the promoter and intron 1 regulatory sequences are required for the vernalization response. According to this model, mutations in either one of these two regulatory sites would be sufficient to eliminate or reduce the vernalization response. A similar coordinated regulation of the vernalization response has been reported for the *Arabidopsis* MADS-box gene *FLC*. Sequence elements within the *FLC* promoter and intron 1 are both required for the initial down-regulation of the *FLC* gene by vernalization (Sheldon et al. 2002).

The presence of the intron 1 deletion in barley *VRN-H1* was associated with a complete elimination of the effect of allelic variation in *Vrn-H2* on flowering time ($P = 0.61$). This result differs from the epistatic interactions observed between *VRN-A*^{m}*2* and *VRN-A*^{m}*1* in *T. monococcum* (Tranquilli and Dubcovsky 2000). In the *T. monococcum* study, a significant effect of *Vrn-A*^{m}*2* on flowering time ($P < 0.0001$) was detected within the dominant *Vrn-A*^{m}*1* class. One possible explanation for this difference is that the interaction between *VRN-1* and *VRN-2* may be affected more by the deletion in intron 1 (dominant *VRN-H1*) than by the deletion in the promoter region (dominant *Vrn-A*^{m}*1*). However, these experiments were done in two different genera and it is not possible to rule out other explanations.

The putative critical region in *VRN-1* intron 1 could be restricted to a 2.8 kb region by combining information from the overlapping deletions and the patterns of sequence conservation among recessive alleles in wheat and barley (Fig. 1). The first 440 bp of this 2.8-kb region showed the highest sequence conservation between wheat and barley, suggesting that this region may include some conserved regulatory elements. Biochemical and/or transgenic experiments will be necessary to identify the putative vernalization-responsive regulatory regions.

Frequencies of different *Vrn-1* allele combinations

Different dominant *Vrn-1* alleles in wheat have different effects on vernalization requirement and flowering time. For example, the presence of the dominant *Vrn-A1* allele results in complete elimination of the vernalization requirement, whereas the presence of the dominant *Vrn-D1* allele is associated with some residual vernalization requirement (Halloran 1967; Maystrenko 1974; Kato and Yamagata 1988). Spring varieties including the dominant *Vrn-A1* allele are usually earlier than those including only combinations of the dominant *Vrn-B1* and *Vrn-D1* alleles (Stelmakh 1993).

These different effects of the *VRN-1* alleles on vernalization requirement might affect their adaptive value in different environments. The molecular markers developed in this and previous studies (Yan et al. 2004a) will facilitate rapid characterization of large wheat and barley germplasm collections, providing insights into the adaptive value of the different alleles and allele combinations.

The analysis of the frequencies of the *Vrn-1* alleles in spring varieties grown in Argentina and California provides an example of this application. The *VRN-1* allele frequencies were similar in these two collections, but they differed significantly from the frequencies reported for a collection of varieties from Canada and the USA and for a large worldwide collection (Table 4). The Argentine and Californian varieties showed a lower frequency of the dominant *Vrn-A1* allele and a higher frequency of the dominant *Vrn-D1* allele relative to the worldwide collection (Table 4). This result agrees with the general trend reported by Stelmakh (1998): a decrease of the dominant *Vrn-A1* and *Vrn-B1* alleles and an increase of the frequency of the dominant *Vrn-D1* allele in regions closer to the equator.

In the cold regions of the world, spring varieties are planted in the spring to avoid cold winter temperatures. In contrast, in temperate regions such as Argentina and California, spring wheat varieties are sown in the winter to take advantage of winter rains. It is tempting to speculate that the different *VRN-1* allele frequencies in Argentina and California compared to the worldwide collection might reflect different performances of certain alleles or allele combinations in spring-sowing versus fall-sowing regions.

Comparison of the observed and expected frequencies for the different *VRN-1* allele combinations might also be a useful tool for generating hypotheses concerning the best allele combinations for a specific environment. For example, in Argentina and California,

allele combinations including the three dominant alleles have an 8–9% lower frequency than expected based on the individual allele frequencies from these regions. Also, the observed frequency of varieties carrying only the dominant *Vrn-A1* allele is 7–10% higher than expected in both regions. The molecular markers developed in this study will facilitate the development of the *VRN-1* isogenic lines necessary to test these hypotheses.

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