

The New Wheat Vernalization Response Allele *Vrn-D1s* is Caused by DNA Transposon Insertion in the First Intron

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Abstract Vernalization requirement in hexaploid wheat is largely controlled by a series of homoeologous *VERNALIZATION* (*Vrn*) genes, *Vrn-A1*, *Vrn-B1*, and *Vrn-D1*. The sequence of the promoter area and first intron of *Vrn-D1* were analysed in 77 hexaploid accessions, representing five wheat species (*Triticum compactum*, *Triticum sphaerococcum*, *Triticum spelta*, *Triticum vavilovii*, and *Triticum macha*) sampled from different ecogeographic areas within 35 countries. Polymorphism was detected in the *Vrn-D1* promoter, resulting in a new *Vrn-D1* haplotype (named here, Hap-8T). Analysis of *Vrn-D1* intron-1 sequences revealed a novel insertional mutation in a subset of *T. spelta* and *T. compactum* accessions. This mutant allele was designated *Vrn-D1s*. Analysis of the 844 bp insertion revealed it to

be a novel transposable DNA element (named DTA_Chimera_KF800714) not previously described in *Triticum*, belonging to the *hAT* superfamily. Finally, we describe a PCR-based assay that discriminates the wild-type *vrn-D1* allele from the *Vrn-D1s* allele. Collectively, the work described here highlights the potential of utilizing minor hexaploid wheat species for the identification of novel alleles of agronomic importance.

Keywords Vernalization · *Vrn-D1* · Hexaploid wheat · Genetic variation · *hAT* transposon · Flowering time

Introduction

The adaptability of wheat to a wide range of environmental conditions is largely determined by allelic diversity in genes regulating seasonal growth habit (*Vrn* genes) and photoperiod response (*Ppd* genes) (Bentley et al. 2013). Allelic variants at the *Vrn* genes, which determine vernalization requirement, are largely responsible for the division of wheat into winter, spring, and facultative types. Previous studies have shown that vernalization response in wheat is controlled by at least four major genetic loci: *Vrn1* (a MADS-box transcription factor), *Vrn2* (a zinc-finger CCT domain gene), *Vrn3* (homologous to the Arabidopsis gene *FLOWERING LOCUS T*), and *Vrn4* (uncloned) (Pugsley 1972; Goncharov 2003; Kippes et al. 2014; Yan et al. 2004b, 2006). Under long-day conditions, *Vrn2* and *Vrn3* are expressed (Yan et al. 2004b, 2006). The *Vrn* genes interact epistatically, with *Vrn2* acting as a direct repressor of *Vrn3* which in turn, upregulates *Vrn1* (Distelfeld et al. 2009; Dubcovsky et al. 2008; Trevaskis et al. 2007; Yan et al. 2006). During vernalization, *Vrn2* transcription is progressively downregulated (Dubcovsky et al. 2006; Yan et al. 2004b). Once vernalization requirement has been fully met, *Vrn2*-mediated repression of *Vrn1* is

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alleviated, with *Vrn1* (or a gene regulated by *Vrn1*) subsequently maintaining the downregulation of *Vrn2* transcription (Loukoianov et al. 2005). The interactions among *Vrn1*, *Vrn2*, and *Vrn3* form a feedback regulatory loop (Distelfeld et al. 2009), in which *Vrn3* is integrator of the vernalization and photoperiod floral pathways (Yan et al. 2006; Trevaskis et al. 2007). Thus, changes in the transcription of any of the *Vrn* genes in this feedback loop result in the modulation of the expression profiles of the other two genes. It is hypothesized that *Vrn-D4* (*Vrn4*) operates upstream (or is a part) of the *Vrn1/Vrn2/Vrn3* positive feedback loop. However, it is not currently known which of these genes is the potential target of *Vrn-D4* (Kippes et al. 2014).

In hexaploid wheat, homoeologous *Vrn1* genes are located on the long arms of the group 5 chromosomes, and named *Vrn-A1*, *Vrn-B1*, and *Vrn-D1* (Law et al. 1975; Iwaki et al. 2002; Yan et al. 2003). The wheat *Vrn1* homoeologues encode MADS-box transcription factors with homology to the *Arabidopsis* meristem identity genes *APETALA1*, *CAULIFLOWER*, and *FRUITFULL* (Yan et al. 2003; Danyluk et al. 2003; Trevaskis et al. 2003; Kinjo et al. 2012). These genes regulate the transition between vegetative and reproductive phase (Ferrandiz et al. 2000; Shitsukawa et al. 2007). The presence of the dominant *Vrn-A1* allele results in complete elimination of vernalization requirement (spring type, earliest heading time), whereas the presence of dominant *Vrn-B1* or *Vrn-D1* alleles alone is associated with residual vernalization requirement (facultative type, intermediate, and the later heading time, respectively) (Stelmakh 1993, 1998). These differences in allelic potency have been shown to correlate with their relative expression level (Loukoianov et al. 2005).

Wheat *Vrn1* has thought to possess multiple regulatory regions (Distelfeld et al. 2009; Diallo et al. 2012). Dominant spring (vernalization insensitive) *Vrn1* alleles are known to be conferred by mutations in the promoter (Yan et al. 2004a; Golovnina et al. 2010; Pidal et al. 2009; Chu et al. 2011) or intron-1 (Fu et al. 2005; Dubcovsky et al. 2006; Santra et al. 2009; Shcherban et al. 2012). These mutations predominantly represent deletions spanning putative *cis*-regulatory regions, and often arose by double-stranded DNA break repair via nonhomologous recombination (Cockram et al. 2007b). It is thought that putative *cis*-regulatory regions in the promoter and intron-1 regulate vernalization response independently (Distelfeld et al. 2009; Zhang et al. 2012).

In previous studies, accessions with spring seasonal growth habit have been identified in wild diploid wheat species such as *Triticum boeoticum* Boiss, *Triticum araraticum* Jakubz., and *Triticum urartu* Thum. (Golovnina et al. 2010). Additionally, there are numerous reports of deletions and insertions within the promoter or intron-1 of *Vrn1* genes in spring cultivars of *Triticum aestivum* and wild tetraploid wheat (Fu et al. 2005; Dubcovsky et al. 2006; Yan et al. 2004a; Golovnina et al. 2010).

The dominant (vernalization nonresponsive) *Vrn-D1a* allele in *T. aestivum* has a large deletion within intron-1 and is associated with spring seasonal growth habit (Fu et al. 2005; Yan et al. 2004a). However, no differences have been found in the promoter sequence compared to the recessive *vrn-D1* allele. A single nucleotide polymorphism (SNP) has been reported in the predicted CArG-box within the promoter region of *Vrn-D1b* alleles found in facultative wheat accessions. However, *Vrn-D1b* has the same deletion in intron-1 as *Vrn-D1a* which decreases vernalization sensitivity (Zhang et al. 2012).

More recently, a large deletion in the first intron of *Vrn-D1* gene was reported in *Aegilops tauschii* Coss (the diploid D genome progenitor). This deletion was associated with a lack of vernalization requirement under long-day conditions (photoperiod >15 h), but had no influence on heading date in field conditions (Takumi et al. 2011). However, there are no reports identifying *Vrn-D1* allelic variants in hexaploid wheat species other than *T. aestivum*.

In this study, we identify sequence variation at *Vrn-D1* in five minor hexaploid wheat species (genome AABBDD), such as *Triticum compactum*, *Triticum sphaerococcum*, *Triticum spelta*, *Triticum vavilovii*, and *Triticum macha*, define their interspecies distribution and identify new allelic variants. Additionally, a novel *Vrn-D1* intron-1 insertion was identified, which represents a member of a new transposon family.

Materials and Methods

Plant Material

A total of 77 accessions representing five hexaploid wheat species from 35 countries and different ecogeographic areas were investigated (Supplementary Table 1). These include domesticated hulled wheat (*T. spelta* L., *T. macha* Dekap, and *T. vavilovii* Jakubz) and free-threshing wheat (*T. compactum* Host, *T. sphaerococcum* Percival). Germplasm was obtained from the National Plant Germplasm System (NPGS, USA) and National Center of Plant Genetic Resources (Ukraine).

DNA Extraction and PCR Amplification

Total DNA from 4-day-old wheat seedlings was extracted following a modified CTAB-method (Doyle et al. 1987). PCR reactions consisted of DNA (~40 ng), 20 mM Tris-HCl (pH 8.8), 10 mM (NH₂)₂SO₄, 2.4 mM MgCl₂, 1 mM KCl, 0.1 % Triton X-100, 250 μM dNTPs, 1.5 % DMSO, 0.5 μM each primer, and 1.2 U *Taq* polymerase. A region of *Vrn-D1* intron-1 was amplified using a primer multiplex consisting of primers Intr1/D/F, Intr1/D/R3, and Intr1/D/R4 (Fu et al. 2005). Additionally, primer pair VRN1INT1_F (Dubcovsky et al.

2006), *Vrn-A1-intr_R1* (this study) were used to further study specific regions of intron-1. A region of the promoter was amplified using previously published primers: VRN1DF and VRN1-INT1R (Yan et al. 2004a). PCR was performed using the following program: denaturation at 94 °C (2 min); 30 cycles of amplification at 94 °C (10 s), annealing (15 s), 74 °C (50 s) per cycle; three cycles at annealing (15 s), 74 °C (50 s), and a final elongation step of 72 °C for 3 min. Further details of all primers, including annealing temperatures, are listed in Table 1. Amplification products were separated on 7 % nondenaturing polyacrylamide gels under 13 V/cm for 3 h. Visualization of amplification products was conducted using a modified silver staining protocol (Budowle et al. 1991).

Cloning and Sequencing of PCR Fragments

PCR amplicons were separated on 1.5 % agarose gels, gel extracted using a GeneJET Gel Extraction Kit (Thermo Scientific), and DNA fragments ligated into pGEM-T (Promega), following the manufacturer's instructions. Plasmid DNA was transformed into JM109 *Escherichia coli* competent cells (L2001, Promega). Transformed cells containing the plasmid carrying an insertion of foreign DNA fragment were detected using white-blue selection on growth medium containing ampicillin, X-Gal, and IPTG. Positive colonies were tested for the presence of cloned PCR products by PCR with universal pUC primers (forward and reverse M13 primers), followed by separation and visualization of PCR products on agarose gels. Plasmid DNA was extracted using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and sequencing PCRs performed using an ABI3700 Bioanalyser (Applied Biosystems), following the manufacturer's instructions. Fluorescently labeled extension products were

precipitated and resuspended in HiDi (Applied Biosystems). The five partial *Vrn-D1* sequences reported in this paper were deposited in GenBank under accessions: KF939526-KF939529 (promoter area), KF800714 (intron-1).

Data Analyses

DNA sequence searches of the *T. aestivum* genome were performed using BLASTn (Zhang et al. 2000) searches of NCBI databases (<http://www.ncbi.nlm.nih.gov/>), project: "454 pyrosequencing of the *Triticum aestivum* (bread wheat) genome to 5X coverage" (Brenchley et al. 2012) (without runs accessions ERR: 16487-16503, 16576, and 16577). Transposable elements were identified by BLASTn analysis of the online databases, TREP (Anderson et al. 2005, <http://wheat.pw.usda.gov/ITMI/Repeats/>) and RepBase (Kohany et al. 2006). For in silico PCR, SNP analysis, DNA repeats searches, and analysis of TIRs-TSDs (terminal inverted repeats–target site duplications) associations, computer programs, and scripts were developed. CpG islands were identified using Geneious 4.8.4 (<http://www.geneious.com>). Multiple sequence alignments were generated using Clustal W (Thompson et al. 1994).

Results

Polymorphism Within the *Vrn-D1* Promoter

Primer pair VRN1DF/VRN1-INT1R flanks of the promoter area (from –772 to 0 bp), exon-1 and part of the first intron (+185 to +248 bp). The forward primer was designed to be specific to the D genome, while the reverse primer was

Table 1 *Vrn1* PCR primer details

Primers	Primer sequence (5'-3')	Annealing temp. (°C)	Expected PCR product size (bp)	Allele	Reference
Intr1/D/F	gttgctgcctcatcaaatcc	64	997	<i>vrn-D1</i>	Fu et al. (2005)
Intr1/D/R4	aatgaaaaggaacgagagcgc		1,841	<i>Vrn-D1s</i>	
Intr1/D/F	gttgctgcctcatcaaatcc	64	1,671	<i>Vrn-D1a</i>	
Intr1/D/R3	ggtcactggtgctgtgctgc				
VRN1INT1_F	atgaaactcggcatgaaga	61	837	<i>vrn-D1</i>	Dubcovsky et al. (2006)
<i>Vrn-A1-intr_R1</i>	ctgtccccgtgagctacttac		841	<i>vrn-A1^a</i>	This study
			1,336	<i>Vrn-A1f</i>	
			1,345	<i>Vrn-A1h</i>	
			1,681	<i>Vrn-D1s</i>	
VRN1DF	cgaccggggcggcagcagtg	61	1,012	Promoter area of <i>vrn-D1</i> gene	Yan et al. (2004a)
VRN1-INT1R	gcaggaaatcgaatcgaag		1,018		
			1,019		
			1,020		

^a Intact intron-1

previously designed to amplify *Vrn-A1* (Yan et al. 2004a). Sequence alignment indicated that the reverse primer anneals to *Vrn1* genes from all three genomes (A, B, and D). Accordingly, this primer combination was optimized for investigation of *Vrn-D1* (Table 1). PAA electrophoresis of the resulting PCR amplicons from the 77 hexaploid wheat accessions studied here (Supplementary Table 1) identified two amplicon sizes—a 1,020-bp fragment (as predicted from the reference *Vrn-D1* genomic sequence) and a 1,019-bp fragment (Fig. 1a). The smaller PCR product was amplified from four *T. spelta* accessions (UA0300304, PI 272573, PI 306550, and PI 323438), as well as from one *T. macha* accession (PI 428178). PCR amplicons from four accessions were cloned and fully sequenced (GenBank: KF939526-KF939529).

Multiple alignments of these sequences with *Vrn-D1* reference sequences (GenBank: AB630962, AB630964, and AY616454) showed several polymorphic features. To further investigate this region of *Vrn-D1*, in silico PCR using GenBank, EMBL, DDBJ, and RefSeq databases was conducted. In silico amplicons of length 1,012, 1,018, and 1,020 bp for *A. tauschii* and 1,020 bp for *T. aestivum* were identified (Supplementary Fig. S1). Among the 1,020-bp amplicons, eight SNPs were detected (G66C, T281C, A394G, C633T, G735A, A747G, G780A, and G868A). However, these polymorphisms were not present in the 1,019-bp amplicons identified here. Additionally, our sequencing did not detect the 8 bp deletion found in the 1,012 bp in silico *A. tauschii* amplicons. All of the 1,019 bp amplicons identified in this study were characterized by deletion of “C” nucleotides at positions 50 and 52 bp, a “T” insertion at 346-site (poly-T region-T-tract) and a “T → C”

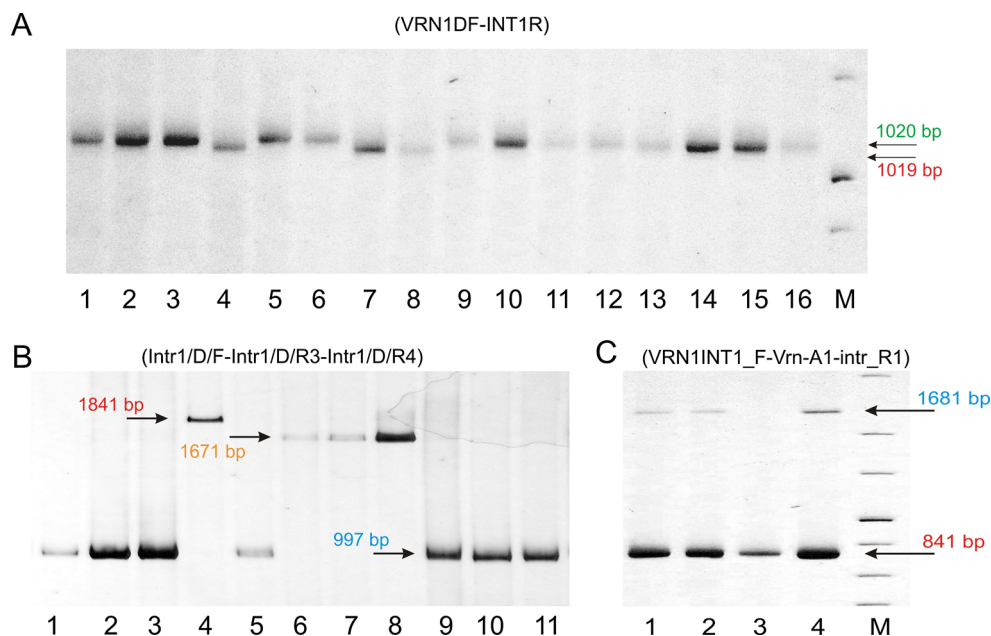
mutation at 30 bp. It is assumed that these polymorphic features caused the observed differences in amplicon mobility in PAA gels. These mutations were not present in sequences previously found in DDBJ/EMBL/GenBank databases and therefore represent a new *Vrn-D1* haplotype (designated Hap-8T, T-tract = (T)₈ whereas an intact sequence (PCR fragment 1,020 bp) designated Hap-7T, T-tract = (T)₇).

Discovery of a New *Vrn-D1* Allele

A multiplex PCR with primers Intr1/D/F, Intr1/D/R3, and Intr1/D/R4 was used to investigate a region of intron-1 thought to span a *cis*-element implicated in the control of vernalization response. Primer pair Intr1/D/F–Intr1/D/R4 spans a region of intron-1 predicted to amplify products of 997 bp in the wild-type (vernalization responsive) allele. However, if the 4,235 bp intron-1 deletion that confers the spring *Vrn-D1a* allele is present, primer pair Intr1/D/F–Intr1/D/R3 results in amplification of 1,671 bp fragments (Fu et al. 2005).

To examine distribution of *Vrn-D1* intron-1 allelic variation, multiplex PCR was performed using the 77 accessions that encompass five hexaploid wheat species. Electrophoretic separation of PCR products identified intron-1 length polymorphisms, resolving fragments of 997 bp (identified in 66 accessions, *vrn-D1*), 1,671 bp (7 accessions, *Vrn-D1a*), and 1,841 bp (4 accessions, not previously characterized) (Fig. 1b; Supplementary Table 1). This indicates that while carriers of the recessive wild-type winter allele (*vrn-D1*, 997 bp) and dominant spring allele (*Vrn-D1a*, 1,671 bp) are present in the accessions investigated, a new allelic variant of the *Vrn-D1* gene involving a size polymorphism within intron-1 has also been identified (1,841 bp). We subsequently

Fig. 1 PCR amplification of *Vrn-1* genes. **M** GeneRuler DNA ladder (100–10,000 bp). **a** Amplification of the promoter region using primers VRN1DF/VRN1-INT1R-4, 7, and 8 identify the novel haplotype Hap-8T (1,019 bp); Hap-7T (1,020 bp)-1-3, 5, 6, and 9-16. **b** Multiplex-PCR targeting *Vrn-D1* intron-1-wild type 1-3, 5, 9-11 (no deletion, amplicon=997 bp); spring *Vrn-D1a* alleles 6-8 (1,671 bp); novel *Vrn-D1s* allele (1,841 bp) 4. **c** Multiplex PCR of the *Vrn-D1s* and *Vrn-D1a* accessions targeting *Vrn-A1* and *Vrn-D1-vrn-A1/Vrn-D1s* 1, 2, and 4 (841/1,681 bp); *vrn-A1/Vrn-D1a-3* (*vrn-D1* null allele)



deconvoluted the multiplex design, and performed PCR analysis using two primer pair combinations (Intr1/D/F, Intr1/D/R3 and Intr1/D/F, Intr1/D/R4). This found the novel 1,841 bp fragment to be amplified by primer pair Intr1/D/F, Intr1/D/R4, indicating that an insertion occurred within intron-1 of the wild-type recessive *vrn-D1* allele. Amplicons with length of 1,841 bp from *T. spelta* (PI 348700) were cloned and sequenced (GenBank accession KF800714). Sequence alignment showed this novel variant includes an 844 bp insertion in *Vrn-D1* intron-1 (position +1,044; from 859 to 1,702 bp, counting from the start of intron-1 in Triple Dirk C (GenBank accession AY747606)). As the novel 1,841 bp amplicon was identified predominantly in *T. spelta*, this new putative *Vrn-D1* spring allele was designated *Vrn-D1s*.

Finally, primer pair VRN1INT1_F (Dubcovsky et al. 2006), *Vrn-A1*-intr_R1 (this study), for which sequence alignments show anneal to *Vrn-A1* and *Vrn-D1*, were optimised for use here. This primer combination was found to simultaneously amplify intron-1 regions from both *Vrn-A1* and *Vrn-D1* homologues (Fig. 1c). PCR amplicons with length of 837 bp identify the wild-type winter *vrn-D1* allele (no intron-1 deletion), while the 1,681-bp product identifies the novel *Vrn-D1s* allele identified in this study. A product of 841 bp is diagnostic for the absence large *Vrn-A1* intron-1 deletion present in the *Vrn-A1c* allele, while products of size 1,336 or 1,345 bp correspond to spring *Vrn-A^mIf* and *Vrn-A^m1h* alleles, respectively. Null alleles (absence of PCR fragments) for one or both of the loci indicate the presence of spring *Vrn-A1c* and/or *Vrn-D1a* alleles.

Discovery and Classification of a Novel Transposon Family

Sequence analysis of the novel 844 bp *Vrn-D1* intron-1 insertion identified three regions with high GC content (>60 %), localized to regions 20–191 bp (high GC region 1 (HGC-1)), 304–510 bp (HGC-2) and 759–844 bp (HGC-3). CpG islands were identified in HGC-1 and HGC-3, but not HGC-2 (Fig. 2). HGC-1 and HGC-3 were also found to contain many repeated sequences (direct, tandem, reverse, and inverted) of various lengths (up to 17 bp).

The 844-bp insertion was used as a query for BLASTn searches of DDBJ/EMBL/GenBank databases. Regions 1–167 and 681–836 bp (which corresponds to HGC-1 and HGC-3, respectively) were found to be present as multiple copies in numerous cereal genomes (*Triticum*, *Aegilops*, and *Hordeum*) with high degree of sequence similarity (>75 %). Furthermore, a highly similar sequence (99 %) was identified, localized on short arm of *T. aestivum* chromosome 4D (IWGSC_CSS_4DS_scaff_2292513:596-1432). However, these regions have not been annotated. It has been suggested that HGC-1 and HGC-3 appear to represent parts of transposable DNA element (TE), not previously described in the *Triticum* genome.

Further sequence analysis of the *Vrn-D1* insertion identified a specific motif of 11 bp, located at positions 1 bp (in a forward orientation) “TAGGACTGGAC” and 826 bp (inverted orientation) “GTCCAGCCCTA”. This short terminal inverted repeat (TIR) is flanked by an 8-bp target site duplication (TSD): “GTGCTGGC” (Figs. 2 and 3). Upstream of the 3'-TIR, sequence analysis identifies a conserved pentameric subterminal repeat (STR): (GCTCG)₄. However, sequence alignment has shown that 5'-TIR carries an “A” nucleotide instead of “G” at position 5 (5'-TIR-5A and 5'-TIR-5G, respectively) and lacks an adjacent STR (Figs. 2 and 3). Considering the evidence listed above, the *Vrn-D1* insertion identified in this study appears to be a compound, hybrid, or transposon. Following standard TE classification criteria (Wicker et al. 2007), this mobile element was annotated as non-autonomous DNA transposon, belonging to the class II, subclass 1, order TIR, and superfamily *hAT* (designated as DTA_Chimera_KF800714).

Multiple alignment was performed for >500 *T. aestivum* DNA sequences which showed high similarity (>80 %) and which covered >50 % of the insertion sequence. Two conserved regions were identified (positions 1–168 and 681–836), within which regions of increased sequence similarity were identified: positions 1–11 bp (spanning the 5'-TIR) and 806–836 bp (3'-TIR and STR). The reduced sequence conservation within the intervening region (168–681 bp) indicates the existence of multiple transposon types within the family. Sequence alignment showed the TIRs to possess SNPs (5'-TIR-5(A/G) and 3'-TIR-3(C/T)), which appear not to affect the transposition process, as each TIR mutant corresponds to several different TSDs (Fig. 3). Since 5'-TIR and 3'-TIR are mismatched, it is likely that (1) different transposases specific for each TIR are involved or (2) these SNPs are synonymous.

TIRs and TSDs are used as features for the classification of TE families (Wicker et al. 2007). To investigate the TIRs and TSDs within the TE identified here, 80 bp regions at the 5' and 3' ends of the element were used for BLASTn searches of the *T. aestivum* genome. Using a similarity cutoff >80 % identity, 742 and 745 hits to the 5'- and 3'-segment were identified, respectively.

In total, 72, 118, 58, 10, 10, and 1 distinct TSDs identified, associated with 5'-TIR-5A, 3'-TIR-3C (the TIRs found in the *Vrn-D1s* TE identified here), 5'-TIR-5G, 3'-TIR-3T, 3'-TIR-5T, and 5'-TIR-3A, respectively (Supplementary Tables 2 and 3). Thirty-seven TSDs were common for the TIRs identified in the *Vrn-D1s* TE, and only 28 and 4 for TIR haplotype combinations 5'-TIR-3G,5G/3'-TIR-3C,5C and 5'-TIR-3G,5A/3'-TIR-3C,5T, respectively, although these pairs are identical TIRs (Table 2). Matching TSDs were not identified for 5'-TIR-5A/5'-TIR-5G, 3'-TIR-3C/3'-TIR-3T (Table 2; Supplementary Table 4). These data indicate that 5'-TIR-5A and 5'-TIR-5G are commonly found in combination with 3'-TIR-3C, but that they are probably nonsynonymous for

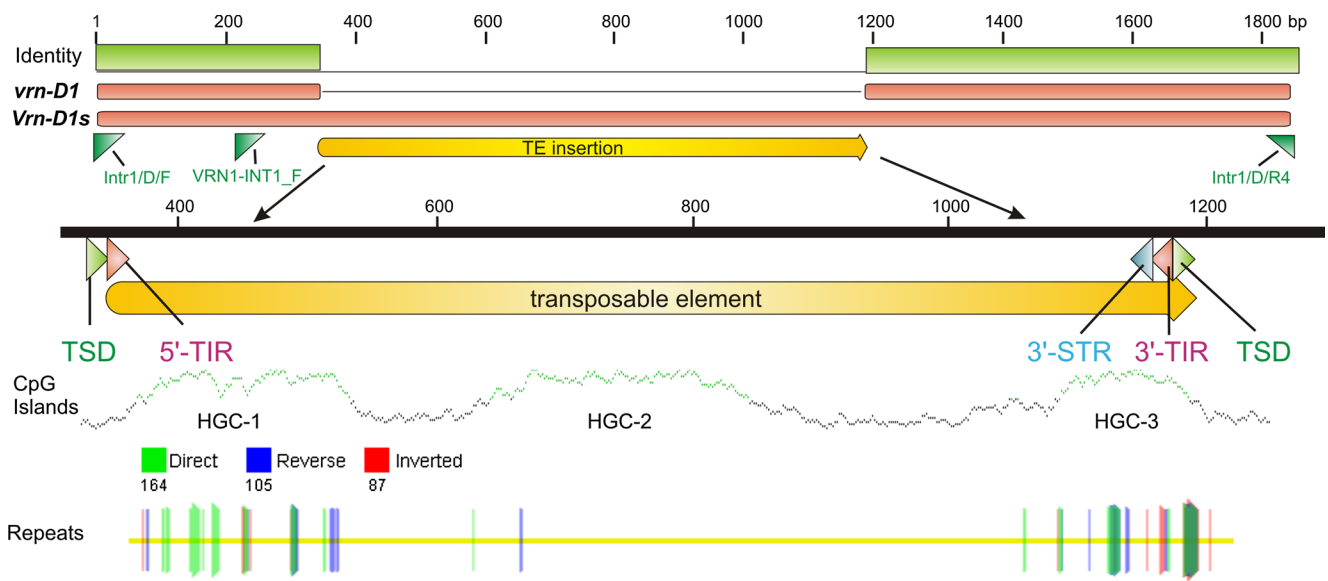


Fig. 2 *Vrn-D1* intron 1, indicating the TE insertion. The wild-type winter allele (*vrn-D1*) and the novel *Vrn-D1s* allele are illustrated, overlaid with TE structure including positions of terminal inverted repeats (TIR), target

site duplications (TSD), and subterminal repeats (STR). Distribution of CpG islands and nucleotide repeats within the TE are also indicated

transposons of a same family since they do not contain common TSDs, but belong to closely related families. 3'-TIR-3C and 3'-TIR-3T in combination with 5'-TIR-5A are nonsynonymous and likely belong to closely related families.

Sequence searches of the TE database RepBase (Kohany et al. 2006) found regions 12–167 bp (encompassing HGC-1) and 684–836 bp (HGC-3) to be highly homologous (>73 % sequence identity) to transposable element belonging to the *hAT* (*hobo/Ac/Tam*) superfamily. Regions 12–167 bp showed 73 % similarity to the maize DNA transposon hAT-N16_ZM, although it lacked regions homologous to the 5'-TIR identified in the *Vrn-D1s* TE. Regions 681–836 bp showed an overall sequence similarity of 75 % to the *Oryza sativa* Ac-like non-autonomous DNA transposon, DS-RICE3N. Within this region, the 3'-STR and 3'-TIR showed 85 % similarity to the rice TE, with the wheat 3'-TIR sequence aligning to the first nucleotide position in DS-RICE3N. However, the DS-

RICE3N TIR has a two-nucleotide mismatch relative to the wheat 3'-TIR investigated here. Additionally, no common TSDs were identified for these TIRs. Accordingly, we conclude that these transposons belong to different, but closely related families. BLASTn searches of the TREP TE database did not identify any homologous transposons. According to established nomenclature (Anderson et al. 2005), this new family of non-autonomous short transposons with different 5'- and 3'-TIRs was designated, as “Chimera”.

Discussion

Defining allelic variation in hexaploid wheat species related to *T. aestivum* is of importance as they are used by plant breeders as a source of agronomically valuable traits. For example,



Fig. 3 Multiple alignment of transposons with high sequence (80 bp segments) similarity (>80 %). The positions of TIRs, TSDs, and SNPs in 5'- and 3'-TIRs are indicated

Table 2 Number of common TSDs for different 5'-TIR/3'-TIR combinations

TIRs	5'-3G,5A ^a	5'-3G,5G	5'-3A,5A	3'-3C,5C ^a	3'-3C,5T	3'-3 T,5C
5'-3G,5A ^a	–	–	–	37	4	–
5'-3G,5G	–	–	–	28	2	4
5'-3A,5A	–	–	–	1	–	–
3'-3C,5C ^a	–	–	–	–	–	–
3'-3C,5T	–	–	–	–	–	–
3'-3 T,5C	–	–	–	–	–	–

^aOriginal TIRs (found in the *Vrn-D1s* TE)

hexaploid wheat species as *T. spelta*, *T. vavilovii*, and *T. macha* have been identified as potential donors of resistance genes to stripe and leaf rust (Bariana et al. 2002; Gulyaeva et al. 2002), *Septoria tritici* (Simon et al. 2001), Russian wheat aphid (Nkongolo et al. 1989), Fusarium head blight (Steed et al. 2005; Buerstmayr et al. 2011), etc. Furthermore, systematic investigations may find novel allelic variants at known genes of agricultural importance. In the case of the cereal *Vrn-1* genes, previous surveys of natural variation have identified novel spring and winter alleles, all of which are due to allelic variation within the promoter or intron-1 (Cockram et al. 2007a; Dubcovsky et al. 2006; Fu et al. 2005; Golovkina et al. 2010; Shcherban et al. 2012; Yan et al. 2004a).

For the major vernalization response locus *Vrn-D1*, no promoter sequence differences have been reported between dominant *Vrn-D1* alleles versus recessive *vrn-D1* alleles (Fu et al. 2005; Yan et al. 2004a). However, several mutations (including SNPs and an 8-bp deletion) have previously been identified within winter and spring allelic classes in *T. aestivum* (Golovkina et al. 2010; Takumi et al. 2011; Zhang et al. 2012) and *A. tauschii* (Takumi et al. 2011). Just one SNP has been identified associated with later flowering of spring accessions possessing a deletion in intron-1 (*Vrn-D1b* allele) (Zhang et al. 2012). In this study of 77 accessions of various hexaploid wheat species, several novel *Vrn-D1* promoter mutations were identified. However, as none localize to the predicted *cis*-regulatory regions within the promoter (CArG, VRN, or G boxes), we assume that they do not influence vernalization requirement. PCR fragments of the new Hap-8T haplotype are characterized by increased migration in PAA gels. The observed difference in the amplicon mobility between the Hap-7T and Hap-8T haplotypes cannot be explained by only 1 bp deletion. We assume that the “T” insertion at position 346 bp (thymine-tract region) contributes more to the differences in the electrophoretic mobility between amplicons than other mutations within the Hap-8T haplotype (Supplementary material “T-tract analysis”).

Our analysis of *Vrn-D1* intron-1 revealed a new spring allele (*Vrn-D1s*) due to TE insertion, present in three *T. spelta* accessions (from Spain and England) and one *T. compactum* accession (Turkey). This indicates that the *Vrn-D1s* allele is not specific to a single hexaploid wheat species (*T. spelta*).

The first intron contains a region required to maintain repression of *Vrn1* (von Zitzewitz et al. 2005; Fu et al. 2005; Cockram et al. 2007a; Hemming et al. 2009). Fu et al. (2005) defined a 4.2-kb region of *Vrn1* intron-1 as the vernalization “critical region”. This region (Fig. 4) is defined by the deletions that have a most distal start point (*Vrn-1c* of IL369) and most proximal end (the 3' end of the *Vrn-D1a* deletion) (Fu et al. 2005), within which deletions result in decreased vernalization sensitivity (Fu et al. 2005; Shcherban et al. 2012). It has been hypothesized that the “critical region” contains a binding site for a putative repressor that is downregulated by vernalization (Fu et al. 2005; von Zitzewitz et al. 2005) or encompass a region implicated in maintaining chromatin in a repressive state (Diallo et al. 2012; Oliver et al. 2009). In contrast, the presence of deletions or TE insertion downstream of the “critical region” do not affect vernalization requirement (Fu et al. 2005).

Insertions of TEs in *Vrn-1* intron-1 (the dominant *Vrn-A^mIf* and *Vrn-A^m1h* alleles) are associated with active expression of *Vrn1* in the absence of vernalization, despite being located 200 bp upstream from the proposed “critical region” (Fig. 4) (Dubcovsky et al. 2006). Similarly, TE insertion within this region of barley *Vrn-H1* intron-1 is also associated with vernalization insensitivity (Cockram et al. 2007a, b). Furthermore, it has been shown in *Arabidopsis* that intron-1 TE insertion within MADS-box genes can affect regulatory regions adjacent to the insertion point (Liu et al. 2004). Collectively, these results suggest that the “critical region” should be extended further in the 5' direction.

Mutations within intron-1 of the homoeologous *Vrn1* genes are reported to be more effective in suppressing the effect of *Vrn2* on flowering time than the allelic variation in the promoter region (Dubcovsky et al. 2008), resulting in the induction of *Vrn1* independently of *Vrn2* expression (Dubcovsky et al. 2005; Hemming et al. 2008; Pidal et al. 2009). This is supported by the presence of 18 independent intron-1 InDel mutations associated with a spring seasonal growth habit in wheat and barley (Cockram et al. 2007a, b; Fu et al. 2005; Dubcovsky et al. 2006; Hemming et al. 2009; Santra et al. 2009; Shcherban et al. 2012; von Zitzewitz et al. 2005). However, it should be noted that the influence of the *Vrn-D1s* allele on phenotype has not been formally defined here. According to passport data, the four accessions carrying the

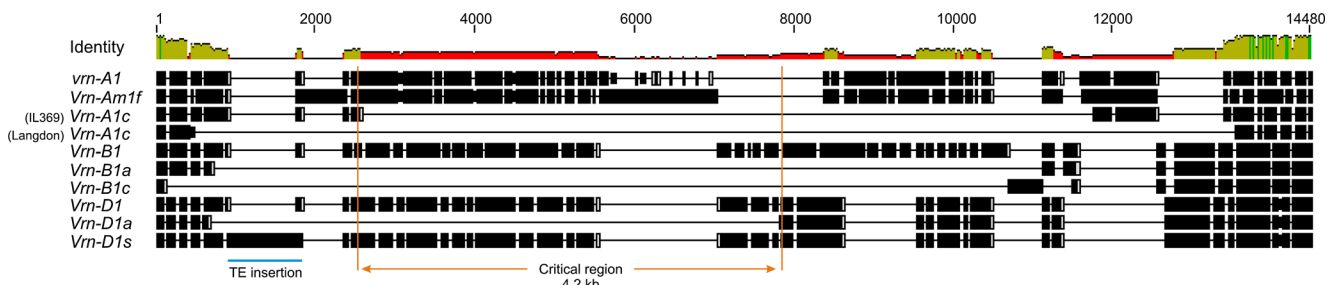


Fig. 4 Alignment of wheat *Vrn1* intron-1 genomic DNA sequences. Alleles that carry large deletions (*Vrn-A1c* (type IL 369, Langdon), 5,504 and 7,222 bp; *Vrn-B1a*, 6,850 bp; *Vrn-B1c*, 6,850 and 820 bp;

Vrn-D1a, 4,235 bp) or TE insertion (*Vrn-A^mIf*, 493 bp) associated with spring alleles are illustrated. The TE insertion in intron-1 of *Vrn-D1s* and the vernalization “critical region” are shown

TE insertion are all characterized as spring wheat. TE insertion in *Vrn-D1s* is located 87 bp upstream from the TE insertions in *Vrn-Am1f* and *Vrn-Am1h*. Further investigation of influence the TE insertion in *Vrn-D1s* on vernalization sensitivity will allow the borders of “critical region” to be established with greater precision.

Sequence analysis revealed the *Vrn-D1* intron-1 insertion identified in this study represents a new non-autonomous transposable DNA element belonging to the *hAT* superfamily. This superfamily includes the transposase-like genes that possess 8 bp TSDs and relatively short TIRs (5–27 bp) (Kempken et al. 2001), and mobilize via the classical “cut-and-paste” mechanism. Members of the *hAT* superfamily have been observed in most eukaryotic lineages, including fungi, animals and plants (Rubin et al. 2001). The assumption that the TE identified here is non-autonomous is based on the following arguments: firstly, most autonomous *hAT* family members include a transposase coding region with length of several thousands of kilobyte (Kempken et al. 2001). Secondly, multiple sequence alignment showed that central region 168–680 varies greatly between TEs with identical TIRs. Finally, similarity searches has not identified for this region of transposase encoding homologues.

According to the “80–80–80” rule (Wicker et al. 2007), two TEs belong to the same family if they share 80 % (or more) sequence identity in ≥ 80 % of their coding or internal domain, or within their terminal repeat regions, or in both. To avoid misclassification, it is recommended that segments >80 bp are analyzed. Therefore, compound or hybrid elements must be annotated segmentally, using minimum segment lengths of 80 bp (Wicker et al. 2007). Following these conventions, similarity searches using the 5'- and 3'-TE regions in the *T. aestivum* genome showed that TIRs of associated with this transposon contain nonsynonymous SNP mutations. It is assumed that a characteristic of transposons from this family is a specific combination of 5'-TIR and 3'-TIR that are insignificantly different at specific sites. Compound transposons can arise from the juxtaposition of different individual TIRs or sequences resembling the TIRs of autonomous transposons (MacRae et al. 1992; Tsubota et al. 1991). This suggests that the TE identified here consists of remains (regions 1–167 and

681–836 bp) belonging to different transposons of closely related families. Furthermore, based on the strong conservation of TIRs, and their association with multiple TSDs, we conclude that the members of this TE family are involved in transposition.

Identification of the novel *Vrn-D1* intron-1 TE insertion here will allow future studies to quantify its effect on vernalization response and early flowering, alone and in combination with spring alleles at the *Vrn-A1* and/or *Vrn-B1* loci. The diagnostic PCR assay for the presence of the *Vrn-D1* allele developed here will help track this allele in future studies, and broaden the toolkit of diagnostic molecular assays available for *Vrn1* alleles in temperate cereal species (Cockram et al. 2009; Fu et al. 2005; Shcherban et al. 2012; Yan et al. 2004a).

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