VRN-1 gene- associated prerequisites of spring growth habit in wild tetraploid wheat *T. dicoccoides* and the diploid A genome species

Andrey B Shcherban*, Kseniya V Strygina and Elena A Salina

**Abstract**

**Background:** In order to clarify the origin of spring growth habit in modern domesticated wheat, allelic variability of the VRN-1 gene was investigated in a wide set of accessions of the wild tetraploid species *Triticum dicoccoides* (BBAA), together with diploid species *T. monococcum*, *T. boeoticum* and *T. urartu*, presumable donors of the A genome to polyploid wheats.

**Results:** No significant variation was found at the VRN-B1 locus of *T. dicoccoides*, whereas at VRN-A1 a number of previously described alleles were found with small deletions in the promoter (VRN-A1b, VRN-A1d) or a large deletion in the first (1st) intron (VRN-A1L). The diploid A genome species were characterized by their own set of VRN-1 alleles including previously described VRN-A1f and VRN-A1h alleles with deletions in the promoter region and the VRN-A1ins allele containing a 0.5 kb insertion in the 1st intron. Based on the CAPS screening data, alleles VRN-A1f and VRN-A1ins were species-specific for *T. monococcum*, while allele VRN-A1h was specific for *T. boeoticum*. Different indels were revealed in both the promoter and 1st intron of the recessive VRN-A1u allele providing specific identification of *T. urartu*, the proposed donor of the A genome to modern wheat. We found that alleles VRN-A1b and VRN-A1h, previously described as dominant, have either no or weak association with spring growth habit, while in some diploid accessions this habit was associated with the recessive VRN-A1 allele.

**Conclusions:** Spring growth habit in diploid wheats was only partially associated with indels in regulatory regions of the VRN-1 gene. An exception is *T. monococcum* where dominant mutations in both the promoter region and, especially, the 1st intron were selected during domestication resulting in a greater variety of spring forms. The wild tetraploid *T. dicoccoides* had a distinct set of VRN-A1 alleles compared to the diploids in this study, indicating an independent origin of spring tetraploid forms that likely occurred after combining of diploid genomes. These alleles were subsequently inherited by cultivated polyploid (tetraploid and hexaploid) descendants.

**Keywords:** Allelic diversity, Vernalization, VRN-1 gene, Promoter, First intron, Tetraploid, Diploid, *Triticum*
domestication, employing morphological, cytological [6,10] and molecular marker analysis techniques [7,11-14], many questions remain unanswered with respect to the phylogenetic relationships between diploid wheat species and between diploids and their polyploid descendants.

Adaptability of polyploid wheat to a wide range of environments has been at least partially facilitated by allelic diversity in VRN genes regulating growth habit and flowering time [15]. Wheats are categorized into two major forms: those requiring vernalization or exposure to cold to accelerate flowering (winter forms) and those which do not require vernalization (spring forms). Vernalization prevents floral initiation during winter, thereby protecting sensitive floral meristems from freezing temperatures. In recent years, researchers have significantly advanced our understanding of the molecular mechanisms of vernalization in wheat. The genomic locations and sequences of three vernalization genes (VRN-1, VRN-2 and VRN-3) responsible for spring/winter growth habit have been determined [16-18].

The VRN-1 gene is proposed as the main initiating factor of the regulatory cascade initiating flowering [16,19]. VRN-1 encodes a MADS-box transcription factor which controls the transition of the vegetative shoot apical meristem to the reproductive phase [20,21]. In vernalization-requiring cereals, VRN-1 expression is induced by vernalization, with the level of expression being dependent on the length of cold exposure [22]. VRN-1 downregulates the floral repressor VRN-2, and allows long-day induction of the floral activator VRN-3 to accelerate subsequent stages of floral development [17,19].

Changes in the growth habit of winter wheat to spring wheats are primarily due to dominant mutations in regulatory regions (promoter or intron 1) of VRN-1 [15]. VRN-A1 alleles of the hexaploid or common wheat T. aestivum L. (2n = 42, BBAAADD) and the tetraploid T. turgidum L. (2n = 28, BBAA), have so far been identified containing nucleotide deletions as well as insertions of mobile elements as both regions [23,24]. By contrast, dominant mutations in VRN-B1 and VRN-D1 genes are predominantly caused by large deletions in the 1st intron [24]. Recently, a novel VRN-B1 allele was identified as having a deletion coupled with sequence duplication within intron 1 [25-27].

Most of the wild Triticeae species have a winter growth habit, suggesting that the recessive VRN-1 allele is the ancestral form. By contrast, there are many cultivated polyploid wheats with a spring growth habit and with at least one dominant VRN-1 allele [28]. Reports demonstrate that different combinations of dominant and recessive VRN-1 alleles (VRN-1 genotype) significantly affect the time of flowering [29,30]. Spring growth habit determined by dominant VRN-1 alleles could be either inherited from ancestral diploids or could result from selection of independent mutations appearing during the adaptation to different environments after domestication. To resolve this issue, a large scale screening of VRN-1 polymorphisms among both diploid and polyploid wheat species is required. To date, a limited number of accessions of diploid and tetraploid progenitors of T. aestivum have been examined [16,23,24,31,32].

In the present study, molecular variability of VRN-1 genes was analyzed based on a representative set of accessions of wild tetraploid wheat and diploid A genome species. The main objective was to identify and characterize different alleles of VRN-1 genes and to analyze their distribution among accessions. We also examined the influence of different alleles and their combinations on vernalization requirement. This analysis sheds light on the evolution of VRN-1 genes in diploids and during the first stages of wheat polyploidization. It contributes to our understanding of the phylogenetic relationships between diploid and polyploid wheat species and the evolutionary history of modern domesticated wheat.

Results

VRN-1 allelic variability in wild tetraploid wheat T. dicoccoides

Promoter region

Vernalization sensitivity in tetraploid wheat T. dicocoides is controlled by alleles at the 2 homoeologous loci, VRN-A1 and VRN-B1. The specific primers Vrn1AF and Int1R were used in order to identify variation in the promoter region of VRN-A1 locus as described by Yan et al. [23] (Table 1). Almost all of the 80 studied accessions of T. dicoccoides yielded a PCR product of approximately 0.7 kb (Figure 1a). To further analyse these PCR products, we digested them with a frequently-cutting restriction endonuclease Msp I. The restriction patterns could be divided into two types (Figure 1b). The first type, characteristic of the vast majority of the studied accessions, contained two major fragments of ~140 and 200 bp long. The second type was found in only 3 of the 80 T. dicoccoides accessions and was characterized by a fragment of approximately 120 bp in place of the 140 bp band. To further analyze allelic variation at the VRN-A1 promoter, we selected a set of accessions representing both restriction patterns and sequenced the PCR products obtained with VrnA1F/Int1R primers (Table 2).

The VRN-A1 promoter sequences of accessions IG 113301, IG 139189, IG 46297, k-5198, belonging to the first restriction pattern type (Figure 1b) were completely identical to the known sequence of the recessive VRN-A1 allele from T. dicoccoides (AY747598) and contained an Msp I restriction fragment of 138 bp. VRN-A1 promoter sequences of all polyploid wheats both studied here and from databases contained the 8 bp insertion and an additional Msp I site characteristic of the respective
Table 1 PCR markers for determining the presence of different alleles of VRN-A1, VRN-B1 in diploid and polyploid wheats

| PCR marker          | Name      | Primer (5′—→ 3′)                      | Target allele(s) | Expected product size (bp) | Annealing temp. (ºC) | Reference.       |
|---------------------|-----------|---------------------------------------|-------------------|----------------------------|----------------------|------------------|
| VRN-A1 marker*      | Vrn1AF    | GAAAGGAAAAATTCTGCTCG                   | VRN-A1α           | 876 and 965                | 55.0                 | [16,23,31]       |
|                     | Int1R     | GCAGGAAATCGAATCGAG                    | VRN-A1β, VRN-A1d, VRN-A1f, VRN-A1h | 694, 662, 684, 703       |                      |                  |
|                     |           |                                       | VRN-A1i           | 704                        |                      |                  |
|                     |           |                                       | VRN-A1u           | 705                        |                      |                  |
| T. monococcum VRN-A1 | Indel(−)F | CGCTCTATATTGTGTACACAGG                 | VRN-A1            | 1025                       | 50.0                 | _                |
|                     | Indel(−)R | GGGGACACTATCTGTGAG                    | VRN-A1u, VRN-A1u' | no product                |                      |                  |
| VRN-A1 deletion of 1.4 kb | Intr1/C/F | GCACTTCTAACCCACTAACC                  | VRN-A1u, VRN-A1u' | 1068                      | 56.0                 | [24]             |
|                     | Intr1/AB/R | TCATCATCATCAAGCCAAA                  | VRN-A1u, VRN-A1u' | no product                |                      |                  |
| VRN-A1 insertion of 0.5 kb | Intr 1 | ATCATCTTCCACCAAGG                   | VRN-A1ins         | 1980                       | 50.0                 | _                |
|                     | Intr1insR | AATGAACACGCAACGAAACAG                | VRN-A1             | 1476                       |                      |                  |
| VRN-A1 deletion of 7.2 kb | Ex1/C/F | GTTCATCCACCAGCTGTTGGA           | VRN-A1L            | 522                        | 55.6                 | [24]             |
|                     | Intr1/A/R3 | AAGTAAGACACGAACTGTTGAG     | VRN-B1, VRN-B1u   | 814                        | 55.0                 | [25]             |
| VRN-B1 marker*      | P2        | TATGCACGACACACGCTA             | VRN-B1            | 1149                       | 56.4                 | [24]             |
|                     | P5        | GGCCAACCTCACCCCAAG                | VRN-B1            | 1149                       | 56.4                 | [24]             |

*These diagnostic markers detect allelic variation at the promoter regions. In other cases variation within intron 1 of corresponding genes is detected.

Figure 1 PCR amplification of VRN-1 promoter region. a - PCR amplification with primers Vrn1F/Int1R. b - Msp I restriction digestion of corresponding PCR products. c - PCR amplification with primers VRN1F_F/VRN1F_R followed by Taq I restriction digestion. Accession numbers, species and genotypes are given at the top. The main restriction fragments more than 100 bp in length are indicated by arrows.
VRN-A1u allele of *T. urartu* (see below). Taking into account these features, we designated the promoter sequence of the recessive VRN-A1 allele of *T. dicoccoides* as VRN-A1u’ (Figures 2 and 3; Table 2; Additional file 1).

Accessions IG 46273, IG 46306 and IG 115810 of the second type were identified to be a previously described VRN-A1b allele [23], with a 19 bp deletion in the promoter region as compared to the recessive allele (Figure 2). This deletion created the diagnostic *Msp I* restriction fragment of 119 bp (Figure 3).

Two out of 80 *T. dicoccoides* accessions (IG46277 and IG 46288) yielded a slightly smaller PCR product compared with the other accessions (Figure 1a). They were almost undistinguishable from the accessions of the first type by *Msp I* restriction (Figure 1b). Sequencing showed that these accessions contained a previously described VRN-A1d allele [23]. Compared with the VRN-A1b allele, this allele has an additional 32 bp deletion, generating a diagnostic 147 bp *Msp I* restriction fragment (Figures 2 and 3).

For analysis of the VRN-B1 locus the primers P2/P5 were used to amplify an approximately 0.8 kb region of the promoter sequence (Table 1). No differences were revealed by PCR or subsequent *Msp I* digestion (see Additional file 2). Based on the known VRN-B1 sequences of *T. aestivum* (AY747602-04, AY616453, AY616456), *Msp I* restriction fragments of 494, 104 and 128 bp were expected. We sequenced PCR products of 0.8 kb from 5 randomly selected accessions with diverse origins (see Additional file 1). All five sequences were highly similar to

### Table 2 Allelic variants of VRN-A1 gene in a selected set of accessions of diploid *Triticum* species and tetraploid *T. dicoccoides*

| Species (genome) | Accession number | Growth type (Winter/ Spring) | VRN-A1 structural forms* Promoter | Intron 1 | Alleles** | GenBank Ac.N. | Homologous sequences from GenBank |
|-----------------|-----------------|-----------------------------|-----------------------------------|---------|-----------|-------------|-------------------------------|
| T. monococcum (AA) | TRI 1510 | S | VRN-A1 | + | 0.5 kb | 1.4 kb/7.2 kb | VRN-A1ins | KMS86653 | AY188331 |
| T. boeoticum (AA) | TRI 17025 | S | VRN-A1 | - | 0.5 kb | 149 kb/7.2 kb | VRN-A1vm2 | KMS86657 | AY451745 |
| | TRI 17027 | S | VRN-A1 | - | 0.5 kb | 149 kb/7.2 kb | VRN-A1ins | KMS86653 | AY188331 |
| | TRI 17098 | S | VRN-A1 | - | 0.5 kb | 149 kb/7.2 kb | VRN-A1 | KMS86657 | AY451745 |
| | TRI 17103 | W | VRN-A1 | - | 0.5 kb | 149 kb/7.2 kb | VRN-A1 | KMS86657 | AY451745 |
| T. urartu (AA) | TRI 17117 | W | VRN-A1u | - | 0.5 kb | 149 kb/7.2 kb | VRN-A1u | KMS86659 | GQ482970 |
| T. dicoccoides (BBAA) | IG 113301 | W | VRN-A1u | - | 0.5 kb | 149 kb/7.2 kb | VRN-A1u | KMS86660 | AY747508 |

*In all cases the promoter region was sequenced; insertion and deletions in intron 1 were determined by PCR (see Table 1).**vrn-A1, vrn-A1u, vrn-A1u’- recessive alleles (initial structural forms); VRN-A1- structurally modified (mutated) forms.

VRN-A1u’ allele of *T. urartu* (see below). Taking into account these features, we designated the promoter sequence of the recessive VRN-A1 allele of *T. dicoccoides* as VRN-A1u’ (Figures 2 and 3; Table 2; Additional file 1).

Accessions IG 46273, IG 46306 and IG 115810 of the second type were identified to be a previously described VRN-A1b allele [23], with a 19 bp deletion in the promoter region as compared to the recessive allele (Figure 2). This deletion created the diagnostic *Msp I* restriction fragment of 119 bp (Figure 3).

Two out of 80 *T. dicoccoides* accessions (IG46277 and IG 46288) yielded a slightly smaller PCR product compared with the other accessions (Figure 1a). They were almost undistinguishable from the accessions of the first type by *Msp I* restriction (Figure 1b). Sequencing showed that these accessions contained a previously described VRN-A1d allele [23]. Compared with the VRN-A1b allele, this allele has an additional 32 bp deletion, generating a diagnostic 147 bp *Msp I* restriction fragment (Figures 2 and 3).

For analysis of the VRN-B1 locus the primers P2/P5 were used to amplify an approximately 0.8 kb region of the promoter sequence (Table 1). No differences were revealed by PCR or subsequent *Msp I* digestion (see Additional file 2). Based on the known VRN-B1 sequences of *T. aestivum* (AY747602-04, AY616453, AY616456), *Msp I* restriction fragments of 494, 104 and 128 bp were expected. We sequenced PCR products of 0.8 kb from 5 randomly selected accessions with diverse origins (see Additional file 1). All five sequences were highly similar to
each other and to known VRN-B1 sequences (98–100% homology). Minor variation between individual sequences was found including small deletions of up to 7 bp, however, almost all of this variation was upstream from the conserved region containing putative transcriptional signals (Figure 2). The VRN-B1 sequences yielded in this study were deposited to Genbank under Ac.N. KM586661-65.

1st intron
The primer pair Intr1/C/F and Intr1/AB/R for the 1st intron of VRN-A1 (Table 1; Figure 4) were previously used to confirm the presence of the recessive allele of T. aestivum [24]. This allele contains a 1.4 kb deletion compared with the respective alleles of T. monococcum and T. boeoticum (see below). Almost all the studied accessions of T. dicoccoides yielded a PCR product of approximately 1 kb, indicating the presence of the deletion, except for accessions k-5198, IG 139189 and IG 46297 which gave no products (Figure 5c). Previously, VRN-A1 allele from the tetraploid variety ‘Langdon’ (AY747598) was determined as having a 7.2 kb deletion in intron 1. Using the primers pair Ex1/C/F and Intr1/A/R3 for detection of this allele (Table 1; Figure 4), the three T. dicoccoides accessions generated a PCR product of about 0.5 kb (Figure 5d) identical to the corresponding sequence from ‘Langdon’. Here, we refer to this allele as VRN-A1L (Figure 4; Table 2).
The pair of primers Intr1/B/F and Intr1/B/R4 was designed as a positive control for the absence of large mutations (deletions or insertions) in intron 1 of the VRN-B1 locus [24] (Table 1). Using these primers, all accessions of *T. dicoccoides* yielded a 1149 bp PCR product indicating the presence of the wild recessive VRN-B1 allele (see Additional file 2).

Thus, tetraploid *T. dicoccoides* displayed structural variation both at the promoter and 1st intron regions of VRN-A1 locus, whereas at VRN-B1 no significant differences were found in the studied accessions. To determine whether this variation originated at the tetraploid level or in diploid donors of the A genome, we conducted an analysis of diploid progenitor species.

**Figure 3** *Msp I* restriction enzyme map of the promoter region of different VRN-1 alleles. Start codon and the 1st exon are indicated by the right-angled arrow flanking the grey rectangle. The positions of specific primers are shown at the top of the diagram. Dashes with black circles indicate *Msp I* restriction sites. Lengths of restriction fragments longer than 100 bp are indicated. Micro-deletions and insertions are shown by the empty and filled triangles, respectively, with sizes indicated. Positions of the CArG box and nucleotide G→C substitution are shown.

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**VRN-1 allelic variability in diploid *Triticum* species**

**Promoter region**

We analysed VRN-1 promoter variability in 160 accessions of the diploid wheat species *T. monococcum*, *T. boeoticum* and *T. urartu* (Additional file 1). As for the tetraploid accessions, the diploids yielded PCR products of ~0.7 kb with primers Vrn1AF and Int1R (Figure 1a). *Msp I* digestion yielded three patterns of restriction (Figure 1b). The first pattern contained bands of 119, 162 and 200 bp, consistent with the known recessive VRN-A1 allele of *T. monococcum* (AY188331). This pattern was characteristic of all 61 accessions of *T. monococcum* and 39 accessions of *T. boeoticum* (Additional file 1). The remaining 20 accessions of *T. boeoticum* yielded a
**Figure 4** Schematic representation of the 1st intron region of different VRN-1 alleles. The positions of specific primers are shown above each scheme. Large deletions and insertions are indicated.

**Figure 5** PCR amplification of VRN-1 intron 1 region. PCR amplification with primers Intr1/Intr1insR (a), Intr1/C/F/Intr1/AB/R (b), Intr1/C/F/Intr1/AB/R (c), Ex1/C/F//Intr1/A/R3 (d). Accession numbers, species and genotypes are given at the top.
pattern with no middle band at 162 bp, and intensive 200 bp band. All accessions of *T. urartu* were distinguished from the other two species by a double band of about 120–130 bp (Figure 1b). To further analyze allelic variation in the VRN-A1 promoter, we selected a subset of accessions representing the three restriction patterns and sequenced the corresponding PCR products obtained with VrnA1F/Int1R primers (Table 2).

Among five selected accessions of *T. monococcum*, four had identical promoter VRN-A1 sequences to the reported VRN-A1 allele (AY188331) (Table 2). Accession TRI 28871 had a 1 bp deletion in a CArG-box motif in the promoter region (Figure 2). This allele has been previously identified in a spring accession of *T. monococcum* as VRN-A1f [16]. CAPS marker screening of all diploid accessions (see Methods) revealed a 112 bp band characteristic of VRN-A1f (Figure 1c) in three more accessions of *T. monococcum* (Additional file 1). Subsequent sequencing confirmed the presence of VRN-A1f in these accessions.

Of seven selected accessions of *T. boeoticum*, three had promoter sequences which were identical to the recessive VRN-A1 allele of *T. monococcum* (Table 2). Sequences of the remaining four accessions were identical to the previously described VRN-A1h allele (GQ451745), which differs from VRN-A1 by a 20 bp deletion near the CArG-box motif (Figure 2). This deletion resulted in a loss of one *Msp* I site (Figure 3), explaining the absence of the 160 bp band and increased staining intensity of the 200 bp band due to superposition of a 198 bp fragment (Figure 1b). We concluded that the 20 *T. boeoticum* accessions showing this restriction pattern carry VRN-A1h, whereas the remaining accessions are likely to have VRN-A1 (Additional file 1).

The five selected accessions of *T. urartu* had an identical VRN-A1 promoter sequence (Table 2), with 98% sequence identity to the respective sequence of *T. monococcum* VRN-AI. Of the few nucleotide substitutions, one (G → C, position –48 from the start codon) resulted in a new *Msp* I restriction site (Figure 2, 3). This explains the appearance of a 130 bp band coupled with the loss of the 160 bp band (Figure 1b). Based on our restriction analysis across the three diploid species, this substitution is specific for *T. urartu*, so we have designated the corresponding allele as VRN-A1u (Additional file 1).

**1st intron**

Alignment of the known VRN-A1 sequences from databases at the 1st intron revealed two large indels (Figure 4). An insertion of 0.5 kb (VRN-A1ins) was found previously in two spring accessions of *T. monococcum* near the 5′ side of intron 1 [31]. A deletion of 1.4 kb located about 4 kb downstream from the start of intron 1 was found in all known VRN-A1 sequences obtained from polyploid wheat species in comparison with *T. monococcum*.

We designed specific primers flanking both indels to study variation at the 1st intron of VRN-A1 in three diploid species (Table 1; Figure 4). The primers Indel(−)F and Indel(−)R allow identification of the VRN-A1 allele of *T. monococcum* without the deletion of 1.4 kb, with an expected PCR product of approximately 1 kb. All accessions of *T. monococcum* and *T. boeoticum* yielded this product, whereas no product was obtained with the *T. urartu* accessions (Figure 5b). The primers Intr1/C/F and Intr1/AB/R flanking the 1.4 kb deletion were used as a positive amplification control for the presence of this deletion in *T. urartu* (Table 1; Figure 4). As for *T. dicoccoides* (see above), all *T. urartu* accessions yielded the 1 kb product. This product was absent for all accessions of *T. monococcum* and *T. boeoticum*, confirming the previous result (Figure 5c). Hence, the 1.4 kb deletion in intron 1 is a specific marker for the *VRN-A1u* (*VRN-A1u*) allele of *T. urartu* and polyploid species with an A-genome (Additional file 1).

Using primer pair Intr1/Intr1insR, 19 accessions of *T. monococcum* generated a 2 kb PCR product, demonstrating that these accessions carried VRN-A1ins (accessions TRI 1510, 17212 in Figure 5a; additional file 1). The remaining 42 *T. monococcum* accessions yielded a fragment of about 1.5 kb indicating the absence of VRN-A1ins. Similarly, no VRN-A1ins was found in any of the *T. urartu* or *T. boeoticum* accessions, excepting a single *T. boeoticum* accession from Europe (which may have been mis-classified) (Additional file 1).

**Evaluation of growth habit in diploid and tetraploid accessions**

To assess the impact of VRN-1 alleles on vernalization requirements in diploid and tetraploid wheats, we selected 26 accessions containing both initial (recessive), or structurally modified forms of VRN-1 genes (Table 2). Noteworthy, in two accessions of *T. monococcum* and 1 accession of *T. boeoticum* the recessive form of VRN-A1 (VRN-A1 promoter, no insertion in intron 1) was associated with spring growth habit, implying an involvement of other genes in the determination of this habit. Four accessions of *T. monococcum* carrying a VRN-A1f allele was defined as a spring wheat. Out of four accessions of *T. boeoticum* carrying the VRN-A1h allele, two had a spring growth habit, while the other two were winter types. All five accessions of *T. urartu* with uniform VRN-A1 sequences were winter types (Table 2).

With respect to the tetraploid *T. dicoccoides*, allele VRN-A1b was associated with winter growth habit in all three accessions containing this allele, whereas VRN-A1d and VRN-A1l were strongly associated with spring habit (Table 2). Accession IG 113301 with a recessive
form of the VRN-A1 gene (VRN-A1u’ promoter, no changes in intron 1) was classified as a winter wheat.

Discussion

Previous studies of VRN-1 allelic variability in wild Triticum species were restricted either by inclusion of a limited number of accessions for analysis [16,23,24,31], or by the use of a single regulatory region (VRN-1 promoter) as a marker [32]. Here we have made an attempt to systematically study these species using a wide set of accessions representing wild tetraploid T. dicoccoides and three diploid A genome progenitor species (Additional file 1).

VRN-1 allelic diversity in diploid A genome species

A CAPS markers designed to the promoter VRN-1 region allowed us to reveal the three most frequent allelic variants in diploids, while avoiding a large scale sequencing approach (Figure 1). The recessive VRN-A1 allele was prevalent among the accessions of T. monococcum and T. boeoticum (Additional file 1; Table 2). Twenty accessions of T. boeoticum contained allele VRN-A1h which was absent from all accessions of T. monococcum. We found the previously described VRN-A1f allele (Figure 2) in four accessions of T. monococcum (Table 2; Additional file 1).

All accessions of T. urartu had a specific restriction pattern with the frequent base cutting enzyme, Msp 1, allowing us to distinguish T. urartu from the other diploid species (Figure 1b). Sequencing determined that the corresponding VRN-A1u allele is highly homologous to the known VRN-A1u alleles of polyploid wheat species. The majority of these alleles contain an 8 bp insertion which is absent in the respective sequences of diploids (Figures 2 and 3), except for a single previously studied sequence from T. urartu (GQ451737). We have classified the latter allele as VRN-A1u’, suggesting that it originated from the basic VRN-A1u allele of T. urartu and was subsequently inherited by polyploid descendants bearing an A-genome, including T. dicoccoides (Additional file 1).

Previously, molecular markers were designed for identification of large deletions in intron 1 affecting vernalization response [24]. Using these and our own markers (Table 1) we found two major alterations occurring at different evolutionary stages of diploid wheats (Figures 4 and 5). The insertion of 0.5 kb may have occurred relatively recently during the evolution of T. monococcum, since it was found only in some accessions of this species (Additional file 1). The deletion of 1.4 kb probably occurred in a progenitor of T. urartu after its divergence from T. boeoticum. The estimated divergence time between T. boeoticum and T. urartu and between T. monococcum and T. boeoticum is 570 000 and 290 000 YA, respectively [33]. Besides T. dicoccoides, the 1.4 kb deletion is characteristic of other polyploid species with an A genome.

Thus, the analysis of both the promoter and 1st intron regions of VRN-1 gene in diploid wheat species allowed us to reveal species-specific alleles including VRN-A1f and VRN-A1ins for T. monococcum, VRN-A1h for T. boeoticum and VRN-A1u for T. urartu. The structural similarity of the latter allele with the respective alleles of wheat polyploids confirmed T. urartu as the donor of the A genome to wheat polyploids.

Tetraploid wheat genetic diversity at VRN-1 locus: the beginning of the formation of a new allelic set

Wild emmer wheat T. dicoccoides as a species is about 360,000 years old, resulting from a spontaneous hybridization event which took place somewhere in the Fertile Crescent, a crescent-shaped area of fertile land in the Middle East [34]. T. dicoccoides belongs to the first cereals domesticated by humans and it is this domestication step which provided the key for subsequent bread wheat evolution [1].

Using the same approach as for the diploid wheats to study T. dicoccoides, we found two previously described alleles VRN-A1b and VRN-A1d with mutations in the promoter region (Figure 2 and 3; Table 2). These alleles occur in different polyploid wheats both tetra- and hexaploid but they have not been found in diploid wheat species [23,32]. We did not find the dominant VRN-A1a allele with insertion of a foldback element in the promoter region amongst the T. dicoccoides accessions (Table 1). This allele is abundant in hexaploid wheat T. aestivum [23,35,36].

PCR analysis of the 1st intron region revealed allele VRN-A1L in three accessions of T. dicoccoides (Figure 4 and 5). This allele is common among cultivated spring varieties of tetraploid T. durum [24,37]. VRN-A1L was not found in the diploid wheat species.

Unlike VRN-A1, locus VRN-B1 displayed no significant variability within either the promoter or 1st intron regions (Figure 2, Additional file 2). Several large mutations, mainly deletions within the 1st intron of VRN-B1 were previously found in spring varieties of T. aestivum [24,25,27,35]. But these mutations have not yet been detected in tetraploid wheat species, and are thus likely to have a later origin.

The analysis of VRN-1 polymorphism in polyploid and diploid wheat accessions showed that the two groups differ with respect to the sets of VRN-1 alleles found among accessions.

Effect of VRN-1 gene structure on growth habit

Diploid wheat species

We determined the impact of VRN-1 alleles on growth habit in a selected set of accessions representing three diploid species of the A genome (Table 2). Out of five tested accessions of T. monococcum, four were spring...
types despite the presence of an intact promoter region at VRN-A1. In two of them spring growth habit was associated with insertion in intron 1 (VRN-A1ins). Totally, we found the VRN-A1ins allele in 31% of T. monococcum accessions (Additional file 1).

Previous data support the importance of intron 1 in the vernalization response and heading time determination, and the proposed key regulatory region has been narrowed to a highly conserved 2.8 kb “vernalization critical region” located near the left side of intron 1 [24]. A number of mutations (deletions, insertions etc.) in this region have been associated with high levels of VRN-1 transcripts and a spring growth habit in different crops [24,26,31,38,39]. It is suggested that these mutations may affect epigenetic chromatin states, resulting in a higher basal level of VRN-1 expression [19,26,40,41].

Dubbinkovs et al. suggested that mutation in CArG-box of the VRN-A1f allele of T. monococcum results in spring habit preventing interaction with unknown repressor dependent on photoperiod [31]. However, we can not discriminate the effect of this allele on vernalization response, because in our material it always occurs in combination with VRN-A1ins (Table 2; Additional file 1).

In two accessions of T. monococcum (TRI 17025, 17973) and one accession of T. boeoticum (TRI 17072) spring growth habit was associated with the wild recessive VRN-A1 allele (VRN-A1; no insertion in intron 1; Table 2). Alignment of VRN-1 sequence from accessions TRI 17072 and TRI 17062 of T. boeoticum (spring and winter types, respectively) showed that the first accession had a mutation that has not been described previously, the substitution T → C coupled with 1 bp deletion about 200 bp downstream from the start of intron 1. It remains unclear whether this minor alteration within “critical region” (see above) is the reason for spring growth habit.

Previous studies of vernalisation genes in wheat have identified loss-of function mutations at the VRN-2 gene, the flowering repressor, which result in up-regulation of VRN-1 regardless of its allelic status. [15,17,31]. We studied 3 above mentioned accessions using previously developed molecular markers for VRN-2 gene [17] and found that accession TRI 17025 of T. monococcum contains a mutated recessive VRN-2 allele (data not presented). We failed to define the reason for spring growth habit in accession TRI 17973 of T. monococcum. In previous study, 6% of accessions of this species were of spring type despite the presence of vrn-1/VRN-2 genotype [17]. Based on these data, it was suggested that genes other than VRN-1 and VRN-2 influence vernalisation response in diploid wheat.

Out of four accessions of T. boeoticum containing VRN-A1f allele and studied for vernalization requirements, two were of spring type, while the other two were associated with the winter habit (Table 2).

One plausible hypothesis has been that the spring growth habit of T. monococcum originated in wild populations of T. boeoticum and was later selected by man through cultivation [32]. However, absence of the VRN-A1f allele in T. monococcum coupled with the presence of specific alleles conferring spring growth habit (VRN-A1f, VRN-A1ins) in this diploid leads us to propose an alternative hypothesis, of selection of independent mutations that appeared in T. monococcum during domestication.

We found no spring forms in T. urartu (Table 2). None of the T. urartu accessions included in this study had changes in the promoter or 1st intron regions of VRN-1 which might confer a spring growth habit (Additional file 1). According to Dorofeev et al. [6], all forms of T. urartu are winter forms. Goncharov [42] found only 2% of T. urartu accessions to be spring types. In another previous study, no differences in the promoter region of VRN-1 relative to the recessive allele were found in four spring accessions of T. urartu [32].

**Tetraploid wheat**

In *T. dicoccoides* we found several VRN-1 alleles which are widely distributed among cultivated wheat polyploids. Allele VRN-A1b is not always associated with spring growth habit in hexaploid wheat lines [43], in our study this allele was associated with winter habit in three accessions of *T. dicoccoides* (Table 2). Allele VRN-A1d influences growth habit more strongly, probably due to an additional deletion affecting the CArG box (Figures 2 and 3). It confers spring growth habit in all accessions of *T. dicoccoides* both studied here and from databases. Allele VRN-A1f previously found in many cultivated tetraploid forms of wheat [37] is also associated with dominant spring type (Table 2).

The question of appearance of spring growth habit in *T. dicoccoides* remains open. This habit could be inherited from ancestral diploids, in particular, from ancestral form of *T. urartu*, precursor of A genome. However, as shown above, *T. urartu* is predominantly winter species. Kato et al. [44] studied geographical variation in the vernalisation requirements of *T. dicoccoides* and found that the distribution of spring forms was sporadic and restricted to warmer areas. The authors suggest that the spring type might have evolved from a previous winter prototype of *T. dicoccoides* as an adaptation to warmer conditions. Our data are consistent with this hypothesis implying that the VRN-1 associated determinants of spring growth habit in polyploid wheat were formed after combining of diploid genomes.

**Conclusions**

In the present study we investigated variability in the promoter and 1st intron regions of the vernalization gene VRN-1 in different accessions of wild tetraploid species
T. dicoccoides and its diploid A genome progenitors. Our results indicated that a number of VRN-1 alleles characteristic of polyploid wheats (VRN-A1b, VRN-A1d, VRN-A1L) are found in T. dicoccoides, while others (VRN-A1a, VRN-B1 alleles) probably arose during later stages of polyploid evolution and were selected during domestication. The occurrence of spring forms in the diploid A genome progenitor species is only partially attributed to VRN-1 variation, since some of these forms contained an intact recessive VRN-1 allele, while others displayed a winter habit despite the presence of a modified VRN-1 allele (VRN-A1h). The most abundant allele conferring spring growth habit in diploids was VRN-A1ins of T. monococcum, containing a 0.5 kb insertion in the 1st intron. A higher variability of VRN-1 loci in polyploid species in comparison with diploids may be explained by the hypothesis that stresses due to allopolyploidization may provoke genome instability [45]. Greater VRN-1 loci variability gives polyploid wheat an advantage in conferring adaptation to a broader range of environments.

Methods

Plant material and DNA extraction

Plant material included 80 accessions of the wild tetraploid wheat species T. dicoccoides (BBAA) and the diploid A genome species T. monococcum, T. boeoticum and T. urartu (61, 59 and 40 accessions, respectively). These accessions were selected from collections in the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany), the ICARDA genbank (Syria) and the Vavilov All-Russian Institute of Plant Industry RAN (St. Petersburg, Russia). Accession numbers, phenotypes and genotypes are given in Additional file 1.

Total genomic DNA was extracted from etiolated seedlings as described by Plaschke et al. [46] with modifications. Leaves (from 5 seeds per accession) were pooled, placed in racked collection tubes (24 per rack) and homogenised directly in extraction buffer using a FastPrep-24 (MP Biomedicals, USA).

PCR

PCR primers reported in Yan et al. [23], Fu et al. [24], Shcherban et al. [25] were used to detect the presence of dominant or recessive alleles of VRN-A1 and VRN-B1 loci in diploid and polyploid wheats (Table 1). To further discriminate different alleles in promoter regions of both loci we digested corresponding PCR products with restriction endonuclease Msp I followed by separation of DNA fragments in 2.3% high resolution agarose gel (HydraGene Co., China). The polymorphic 1-bp deletion (VRN-A1f) was detected as described by Dubcovsky et al. [31]. Using primers VRN1F_F (5′-ACAGCCGGCTATGCTCCAGAC-3′) and VRN1F_R (5′-GGAGGATGGCAGGCACAAATC-3′), the second nucleotide of the reverse primer (underlined T) was mutated to generate a Taq I restriction site for the vrn-A1 allele that was absent in the VRN-A1f allele.

The sequences of the T. monococcum VRN-A1 gene (AY188331, DQ146421, DQ146422, DQ146423) and respective sequences of T. aestivum (AY747600, AY747601) were used to design specific primers flanking an insertion of ~0.5 kb and deletion of 1.4 kb in the 1st intron of VRN-A1 (Table 1; Figure 4).

PCR was performed using a DNA Thermal Cycler 480 (Perkin Elmer Cetus, USA). Reaction mixtures were in a volume of 20 μl containing 50–100 ng of genomic template DNA, 1 ng of each of primer, 0.25 mM of each dNTP, 1x reaction buffer (67 mM TrisHCl, pH 8.8; 2 mM MgCl2; 18 mM (NH4)2SO4; 0.01% Tween 20) and 1 unit Taq polymerase. After initial denaturation at 94°C for 2 min, 35 cycles were run at 94°C for 1 min, 55-61°C (depending on the primer pair used) for 1 min, and 72°C for 0.5-2 min, followed by a final extension at 72°C for 5 min. PCR products were separated on 1% agarose gel, stained with ethidium bromide and visualized under UV light.

Sequencing of PCR products

Amplified DNA fragments were excised following electrophoresis, purified using a QIAquick PCR purification kit (QIAGEN, Germany) and directly sequenced using an ABI PRISM Dye Terminator Cycle Sequencing ready reaction kit (Perkin Elmer Cetus, USA) and corresponding specific primers. Sequencing was performed in an ABI PRISM 310 Genetic Analyzer (Perkin Elmer Cetus). All obtained sequences were deposited to GenBank (Table 2).

Evaluation of growth habit

Growth habits of the diploid and tetraploid accessions containing different VRN-A1 alleles were determined from two replicates in 2013–2014 (Table 2). Seeds were sown in the greenhouse (ICG, Novosibirsk) without vernalization under a long day photoperiod regime. Three months after sowing, when all spring standard varieties had headed, experimental materials were classified as either spring (ear emergence) or winter (no visible ear formation) types.

Additional files

Additional file 1: Polymorphisms at the promoter and 1st intron regions of VRN-A1 gene in X accessions of diploid Triticum species and Y accessions of tetraploid T. dicoccoides.

Additional file 2: Variability at VRN-B1 locus in T. dicoccoides. a- PCR amplification using specific primers P2/P5 to detect variation within the VRN-B1 promoter region in different accessions of T. dicoccoides. b- Msp I restriction digestion of corresponding PCR products. c- PCR amplification with primers Intr1/Bf/Intr1/Sb/H4 to detect the absence of deletions in the 1st intron of VRN-B1. Asterisks mark the accessions for which the VRN-B1 promoter region was sequenced (Genbank: KM586661-65).
Contributing interests
The authors declare that they have no competing interests.

Authors’ contributions
AS designed the study, carried out the molecular and bioinformatics experiments and greenhouse analyses, and prepared the manuscript. KS contributed to the molecular analysis. EAS helped with interpretation of the results and critically revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We are grateful to Dr. A. Böerner and Dr. A. Graner (Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany) for supplying seeds of wheat species. The study of diploid species was done in framework of the State Budget Programme (Project No. 15.531.1), the analysis of T. dicoccoides was supported by the Russian Scientific Foundation (Project No. 14-14-00161).

Received: 5 December 2014 Accepted: 16 March 2015
Published online: 31 March 2015

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