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ORIGINAL ARTICLE

Allelic variation at the *VERNALIZATION-A1*, *VRN-B1*, *VRN-B3*, and *PHOTOPERIOD-A1* genes in cultivars of *Triticum durum* Desf.

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Abstract

Main conclusion **The durum wheat varieties from Ukraine, Russia, and Kazakhstan are characterized by the specific allelic composition of the *VRN* genes that sharply distinguish them from the *Triticum durum* varieties from other countries. For numerous varieties, the *VRN* alleles which previously were not found in tetraploid wheat were identified.**

The ability of wheat to adapt to a wide range of environmental conditions is mostly determined by the allelic diversity within genes regulating the vernalization requirement (*VRN*) and photoperiod response (*PPD*). In the present study, allelic variation in the *VRN1*, *VRN3*, and *PPD-A1* genes was investigated for 134 varieties of *Triticum durum* from different eco-geographic areas. It was shown that varieties from Russia and Ukraine have a specific allelic composition at the *VRN* genes, which in quantity and quality differed from European and American cultivars. A large number of varieties of *T. durum* from Russia carry the dominant *Vrn-A1a.1* allele, previously identified mainly in hexaploid wheat. For some varieties from Eastern Europe and Asia, *Vrn-A1i* and *vrn-A1b.3* recently revealed in wheat were also identified. Polymorphism of the *VRN-B1* promoter region, distinguishing all three variants of this sequence (*VRN-B1.f*, *VRN-B1.s,* and *VRN-B1.m*), was detected. It was found that the dominant *Vrn-B1c* allele is commonly found in varieties of *T. durum* from Russia and Ukraine, but not Europe or USA. Furthermore, many Ukrainian and Russian varieties carry the dominant alleles of the both *VRN-A1* and *VRN-B1* genes simultaneously, while varieties from Europe and America carry the dominant allele of *VRN-A1* alone. Finally, a high frequency of the *Vrn-B3a* allele, which previously was found only in some accessions of hexaploid wheat, was observed for varieties from Ukraine and Russia. It was revealed that the Ukrainian pool of *T. durum* varieties is currently the largest genetic source of the dominant *Vrn-B3a* allele in wheat in the worldwide.

Keywords Cereal · Durum wheat · Genetic variation · Photoperiod response · Vernalization requirement

Introduction

The adaptability of wheat to a wide range of environment conditions is mainly controlled by genes, determining the vernalization requirement (*VRN*) and photoperiod sensitivity (*PPD*). These agronomically valuable traits are important to ensure the flowering of wheat in the most favorable climatic conditions and are widely used in

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modern breeding to obtain high-yielding varieties that are favorable adapted to changes in environmental conditions.

The requirement of fall-planted winter wheat for a prolonged exposure to low temperatures for timely flowering in spring, a process known as vernalization, is an important adaptation protecting sensitive floral meristems from frost damage during the winter. The vernalization requirement of wheat is mainly controlled by four *VRN* genes: *VRN1* (a MADS-box transcription factor) (Yan et al. 2003), *VRN2* (a zinc-finger CCT domain gene—*ZCCT*) (Yan et al. 2004b), *VRN3* (*TaFT*, homologous to the Arabidopsis gene *FLOWERING LOCUS T*) (Yan et al. 2006), and *VRN4* (MADS-box transcription factor, duplication of the *VRN-A1* gene in the short arm of chromosome 5D) (Kippes et al. 2015). During regulation of vernalization response of wheat, the *VRN1*, *VRN2,* and *VRN3* genes interact epistatically to form a feedback regulatory loop in which *VRN3* is the integrator of the vernalization and photoperiod floral pathways [reviewed by Muterko et al. (2015a)]. Regulation of the vernalization response in this way enables the developmental stages of wheat to progress in accordance with the seasonal changes in climate.

Vernalization insensitive varieties with a spring growth habit, determined by the recessive *vrn2* alleles, require deletions or non-functional mutations in all *ZCCT* genes simultaneously and have not been observed in naturally-occurring variations in polyploid wheat (Muterko et al. 2015a). However, allelic variation at the *VRN-B2* locus is associated with a partially dominant effect in tetraploid wheat (Distelfeld et al. 2009). *VRN4* is mapped to chromosome 5 of the D genome, and is not relevant to the analysis of tetraploid wheat with the BBAA genome. Thus, the vernalization requirement of tetraploid wheat is mainly determined by allelic variation at the *VRN1* and potentially *VRN3* genes. There are numerous reports of allelic variation in *VRN* genes in *Triticum aestivum* cultivars and wild tetraploid wheat species. However, no significant investigations were found to identify allelic variation at the *VRN* genes in varieties of *Triticum durum*.

VRN1 genes have been mapped to the long arms of the chromosomes 5 homeologous group (Yan et al. 2003) and encode MADS-box transcriptional factors, which are involved in the transition of the apical meristem from the vegetative to reproductive stages (Danyluk et al. 2003). The dominant *VRN-A1* alleles as a rule result in complete elimination of the vernalization requirement and determine a spring growth habit. The presence of either the dominant *VRN-B1* or *VRN-D1* alleles alone is associated with some residual vernalization requirement and later flowering that indicates a facultative growth habit. Allelic variations at the *VRN1* genes are determined by mutations within the promoter region and intron-1. In hexaploid, wheat dominant *VRN-A1* alleles carrying InDels and/or SNP mutations within the promoter region are most common. In contrast, the dominant *Vrn-A1* alleles in tetraploid wheat, depending on species, carry mutations in promoter region or large deletion within intron-1 (Muterko et al. 2016). Furthermore, in winter, varieties of hexaploid wheat (*T. aestivum*) were identified from one to four copies of the *VRN-A1* gene (Würschum et al. 2015). This copy number variation (CNV) polymorphism was associated with change in vernalization requirement duration and flowering time of wheat (Díaz et al. 2012). The dominant *VRN-B1* alleles are mainly caused by the large deletions within the first intron for both hexaploid and tetraploid wheat (Muterko et al. 2016). However, in the latter study, a polymorphism of the *VRN-B1* promoter region associated with early flowering of spring wheat accessions was also identified (Muterko et al. 2016).

Authentic natural variation for *VRN3* has been found only in B genome (Yan et al. 2006; Chen et al. 2013). The *VRN-B3* gene is the wheat ortholog of the Arabidopsis *FT* (*Flowering locus T*) and being a typical florigene induces flowering (Yan et al. 2006). This gene encodes an RAF kinase inhibitor-like protein acting upstream of *VRN1* in a feedback regulatory loop. The dominant allele *Vrn-B3a* contains a 5.3 kb insertion of a retrotransposon in the promoter region, 591 bp from the transcription initiation site that results in overexpression of *TaFT* and early flowering (Yan et al. 2006). The *Vrn-B3c* allele is also dominant and differs from *Vrn-B3a* by deletions of 20 and 4 bp in the retrotransposon sequence. Herewith, these deletions do not influence *VRN-B3* transcription level and flowering time in accessions with *Vrn-B3c* as compared to *Vrn-B3a* (Chen et al. 2013). The dominant *VRN-B3* alleles are very rare. Furthermore, allelic variation at the *VRN-B3* gene was previously studied for only hexaploid varieties of bread wheat (*T. aestivum*) and has not been investigated for species of tetraploid wheat and particularly for varieties of *T. durum* until now.

The sensitivity of wheat to photoperiod indicates the dependence of flowering on day length. Photoperiod response in wheat is mainly determined by the homoeologous series of *Photoperiod 1* (*PPD1*) genes. Recessive *Ppd1b* alleles (wild type) confer sensitivity to day length, with flowering delayed under short days (SDs, <10 h light), while flowering is promoted under long day (LD) photoperiods (~16 h day lengths). Dominant *Ppd1a* mutants confer photoperiod insensitivity, and result in rapid flowering under both SD and LD photoperiods. A photoperiod insensitive (PI) phenotype in wheat is an important agronomic trait, and for this reason, the use of PI alleles becomes widespread in wheat varieties following the "green revolution" and continues to be widely used globally. In wheat, homoeologous *PPD1* genes are located on the short arms of the group 2 chromosomes

(Beales et al. 2007). *Ppd1a* carry mutations within the promoter region, which cause their overexpression independent of photoperiod. Copy number variation (CNV mutations) also is a cause of PI phenotype (Beales et al. 2007; Díaz et al. 2012), but this has a lesser effect on decreasing photoperiod sensitivity than *PPD1* promoter region deletions. Furthermore, CNV mutants have been identified only for *PPD-B1* in hexaploid wheat of *T. aestivum* and were not shown for varieties of *T. durum*. Although numerous mutations within the *PPD-B1* promoter region have been identified in wild emmer wheat, their phenotypic effect is unknown (Takenaka and Kawahara 2012). Thus, currently, the photoperiod insensitive *PPD-B1* alleles for varieties of *T. durum* are not known.

The photoperiod insensitive *Ppd-A1a.1* (1085 bp deletion) and *Ppd-A1a.4* (648 bp deletion) alleles previously identified in hexaploid wheat species of *T. aestivum* and *Triticum compactum*, respectively (Nishida et al. 2013; Muterko et al. 2015b), while such PI alleles as *Ppd-A1a.2* (1027 bp deletion) and *Ppd-A1a.3* (1117 bp deletion) were found in isogenic wheat lines of *T. durum* (Wilhelm et al. 2009). All these alleles carry deletions of different lengths within the promoter region and are characterized by differing effects on flowering time during growth of plants under a short photoperiod (Wilhelm et al. 2009; Nishida et al. 2013; Muterko et al. 2015b). Furthermore, based on the change in curvature of the DNA fragments of *PPD-A1* that is associated with SNPs in the promoter region, the *Ppd-A1b* can be divided into two haplogroups, AI and AII (Muterko et al. 2015b).

In our recent studies, 21 accessions of *T. durum* from 20 countries of the world, excluding Russia, Ukraine, and Kazakhstan, were analyzed at the *VRN1* and *PPD-A1* genes (Muterko et al. 2015b, 2016). In the present study, we have expanded the subset of cultivars of *T. durum* and focused on previously uncharacterized gene varieties from Russia, Ukraine, and Kazakhstan to find how allelic diversity at the *VRN* and *PPD-A1* genes of these varieties differs from those in varieties from other countries. We conduct investigation of *T. durum* cultivars by the diagnostic DNA markers and also using the early proposed methods of the detection of the recently identified new *VRN-A1* and *VRN-B1* alleles (Muterko et al. 2016), which until now were not analyzed in a large set of soft and durum wheat. Furthermore, our study also includes the analysis of the *VRN-B3* gene, which previously was not investigated in tetraploid wheat species and particularly in cultivars of *T. durum*. Finally, using a specially developed algorithm of hierarchical clustering, the analysis of the obtained allelic combinations of *VRN* genes will be grouped to find specific compositions of these alleles for different centers of wheat cultivation.

Materials and methods

Plant material

A total of 134 *T. durum* varieties from 17 countries, with a focus on Ukrainian and Russian varieties, were investigated (Table S1). Germplasm was obtained from the National Centre for Plant Genetic Resources of Ukraine (to request germplasm of varieties No. 1-75 from Table S1, contact: yuriev1908@gmail.com, ncpgru@gmail.com), Siberian Research Institute for Plant Industry and Breeding (by Vjacheslav Piskarev, piskarev@bionet.nsc.ru; varieties No. 76-122 from Table S1), and Kazakhstan-Siberian Program (by Vladimir Shamanin, shamanin@bionet.nsc.ru; varieties No. 123-134 from Table S1).

DNA extraction and PCR amplification

Total DNA from 4-day-old wheat seedlings was extracted following a modified CTAB method (Doyle and Doyle 1987). PCR reactions consisted of: DNA (~40 ng), 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_2)_2SO_4$, 2.6 mM $MgCl_2$, 1 mM KCl, 0.1 % Triton X-100, 250 µM dNTPs, 1.5 % DMSO, 3 ng/µl each primer, and 0.05 U/µl Taq-polymerase. PCR was performed using the following program: denaturation at 94 °C (2 min); 30 cycles of amplification: 94 °C (10 s), annealing (10 s), 72 °C (50 s or 2 min for amplification of the *VRN-B3* promoter region and *VRN-B1* intron-1) per cycle, 3 cycles: annealing (10 s), 72 °C (60 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.

Polyacrylamide and agarose gel electrophoresis

PCR fragments of the *VRN-A1* and *VRN-B1* first intron and *VRN-B3* promoter region were separated on 6.6 % nondenaturing polyacrylamide (PAA) gels (mono/bis-acrylamide ratio 82:1) in 1.38× TBE buffer (123 mM ionic strength), at room temperature, under 6 V/cm. For the separation of the *VRN-A1*, *VRN-B1*, and *PPD-A1* promoter region amplicons, the polyacrylamide gel electrophoresis (PAGE) was carried out as described previously (Muterko et al. 2016). Agarose gel electrophoresis was performed using 1.5 % agarose in 1× TBE buffer. Visualization of PCR fragments in PAA and agarose gels was conducted using ethidium bromide under UV light.

Sequencing of PCR fragments

PCR fragments were excised from agarose gel and purified on silica spin columns according to the manufacturer's

protocol. Sequencing was carried out with the use of BigDye Terminator v3.1 sequencing kit with the subsequent analysis on an ABI 3130×1 Genetic Analyzer. The partial *VRN-A1, VRN-B1*, and *VRN-B3* sequences reported in this paper were deposited in GenBank under accession numbers: KU738895 (Kharkovskaya 1; *vrn-A1b.3*), KX139167 (GK Basa; *Vrn-A1i*), KX139174 (Bashkirskaya 27; *Vrn-A1a.1*), KX268729 (Zenati 368; *Vrn-A1b.1*), KX139169–KX139170 (Marzaga, Lavina; *Vrn-A1b.6*), KX139168 (Hordeiforme 18567-6; *VRN-B1.m*), KX139171 (Elizavetinskaya; *Vrn-B3a*), KX139173 (Donskaya Elegiya; *Vrn-B1c*), and KX139172 [Kargala 28; *Vrn-A1c* (*Langdon*)].

Data analyses

To perform cluster analysis, an algorithm of the controlled sequential hierarchical clustering was developed (Supplemental Material S2). Undirected graphs generated during

Table 1 PCR primers details

Primers	Primer sequence (5′–3′)	Primer design	Annealing temp. °C	Amplified region	Allelic variant	PCR product size (bp)
VRN1AF	gaaaggaaaaattctgctcg	Yan et al. (2004a)	60	*VRN-A1* promoter	*vrn-A1*	713
VRN1-INT1R	gcaggaaatcgaaatcgaag				*Vrn-A1a.1*	944
					Vrn-A1a.2	924, 944
					Vrn-A1a.3	765
					Vrn-A1b	691
					Vrn-A1d	685
					Vrn-A1e	659
					Vrn-A1f	658
					Vrn-A1i	713
					vrn-A^{m}1	705
					Vrn-A^{m}1 g	681
					Vrn-A^{m}1a	671
					vrn-A^{m}1b	656
Vrn-A1-intr_F	ccgtcgaaaggatcgctactg	Muterko et al. (2016)	60	*VRN-A1* intron-1	*vrn-A1*	541
Vrn-A1-intr_R1	cttgtccccgtgagctacttac					
Ex1/C/F	gttctccaccgagtcatggt	Fu et al. (2005)	56	*VRN-A1* intron-1	*Vrn-A1c* (*Langdon*)	522
Intr1/A/R3	aagtaagacaacacgaatgtgaga				*Vrn-A1c* (*IL369*)	2188
Pr1	tacccctgctaccagtgcct	Shcherban et al. (2012)	58	*VRN-B1* promoter	*VRN-B1.f*	968
Pr2	ggccaaccctacaccccaag				*VRN-B1.s*	958
					VRN-B1.m	965
Ex1/C/F	gttctccaccgagtcatggt	Fu et al. (2005)	58	*VRN-B1* intron-1	*Vrn-B1a*	1091
Intr1/B/R3	ctcatgccaaaaattgaagatga				*Vrn-B1b*	1055
					Vrn-B1c	705
Ex1/C/F	gttctccaccgagtcatggt	Fu et al. (2005)	60	*VRN-B1* intron-1	*vrn-B1*	1531
Intr1/B/R4	caaatgaaaaggaatgagagca					
FT-B-INS-F	cataatgccaagccggtgagtac	Yan et al. (2006)	61	*VRN-B3* promoter	*Vrn-B3a*	1765
VRN4-B-NOINS-R	ctatccctaccggccattag					
VRN4-B-NOINS-F2	gctgtgtgatcttgctctcc	Yan et al. (2006)	61	*VRN-B3* promoter	*vrn-B3*	691
VRN4-B-NOINS-R	ctatccctaccggccattag					
durum_Ag5del_F2	cgtcacccatgcactctgtt	Wilhelm et al. (2009)	56	*PPD-A1* promoter	*ppd-A1b*	452
durum_Ag5del_R2	ctggctccaagaggaaacac				*ppd-A^{m}1b*	456
Ppd-A1proF	gtgtcgcacggattttgctc	Muterko et al. (2015b)	56	*PPD-A1* promoter	*Ppd-A1a.1*	405
durum_Ag5del_R2	ctggctccaagaggaaacac	Wilhelm et al. (2009)			*Ppd-A1a.2*	463
					Ppd-A1a.3	372
					Ppd-A1a.4	806

cluster analysis were visualized using Dendroscope 2.5 (Huson et al. 2007).

Results

Three primer pairs were used to detect the allelic variants of the *VRN-A1* gene (Table 1). Although primer pair VRN1AF and VRN1-INT1R flanks more than 460 bp of the promoter region, exon-1 and part of intron-1 the amplicon length polymorphism until now was found to associate with mutations within promoter region and was not shown for covered transcribed region. This allows us to differentiate more than ten promoter alleles of *VRN-A1* based on the difference in amplicon lengths. Furthermore, the informativeness of some DNA markers was significantly increased by carrying out of PAA electrophoresis under special conditions, allowing the allocation of no less than seven additional allelic variants of *VRN-A1*, and avoiding the sequencing and errors associated with it (Muterko et al. 2016). The presence or absence of large deletions within *VRN-A1* intron-1 was defined using separate PCRs with primer pairs Ex1/C/F, Intr1/A/R3 and Vrn-A1-intr_F, Vrn-A1-intr_R1. The 522 bp fragments, amplified with primers Ex1/C/F and Intr1/A/R3, indicate the *Vrn-A1c* (Langdon type) allele, while 541 bp fragments, which amplified with primers Vrn-A1-intr_F and Vrn-A1-intr_R1, detect the intact *VRN-A1* intron-1.

During investigation of *VRN-A1* allelic diversity, it was found that the great majority (72 %) of *T. durum* varieties with the dominant *VRN-A1* carry 7.2 kb deletion within the *VRN-A1* first intron, which characterises the *Vrn-A1c* (Langdon type) allele (GenBank: KX139172), while only 28 % of varieties were found to carry mutations in the promoter region. The five known allelic variants of the *VRN-A1* promoter region were identified by PCR and sequencing (Figs. 1, 2): *Vrn-A1a.1* (GenBank: KX139174), *Vrn-A1b.1* (GenBank: KX268729), *vrn-A1b.3* (GenBank: KU738895), *Vrn-A1b.6* (GenBank: KX139169–KX139170), and *Vrn-A1i* (GenBank: KX139167). The *Vrn-A1a.1* allele was found exclusively in varieties of *T. durum* from Russia, where it accounted for half of all *VRN-A1* promoter region alleles. Among other *VRN-A1* promoter alleles, *Vrn-A1b.6* was also quite frequent (20 varieties), while the canonical *Vrn-A1b.1* was detected only in four varieties. For the Ukrainian variety of Kharkovskaya 1, which according to passport data has a winter growth habit, the *vrn-A1b.3* allele was identified (Fig. 2). The *Vrn-A1i* allele, which differs from *vrn-A1* by an SNP within the A-tract of the VRN-box (Muterko et al. 2016), was shown for Shirvan 3, Shirvan 5, and GK Basa from Azerbaijan and Hungary, respectively (Fig. 2).

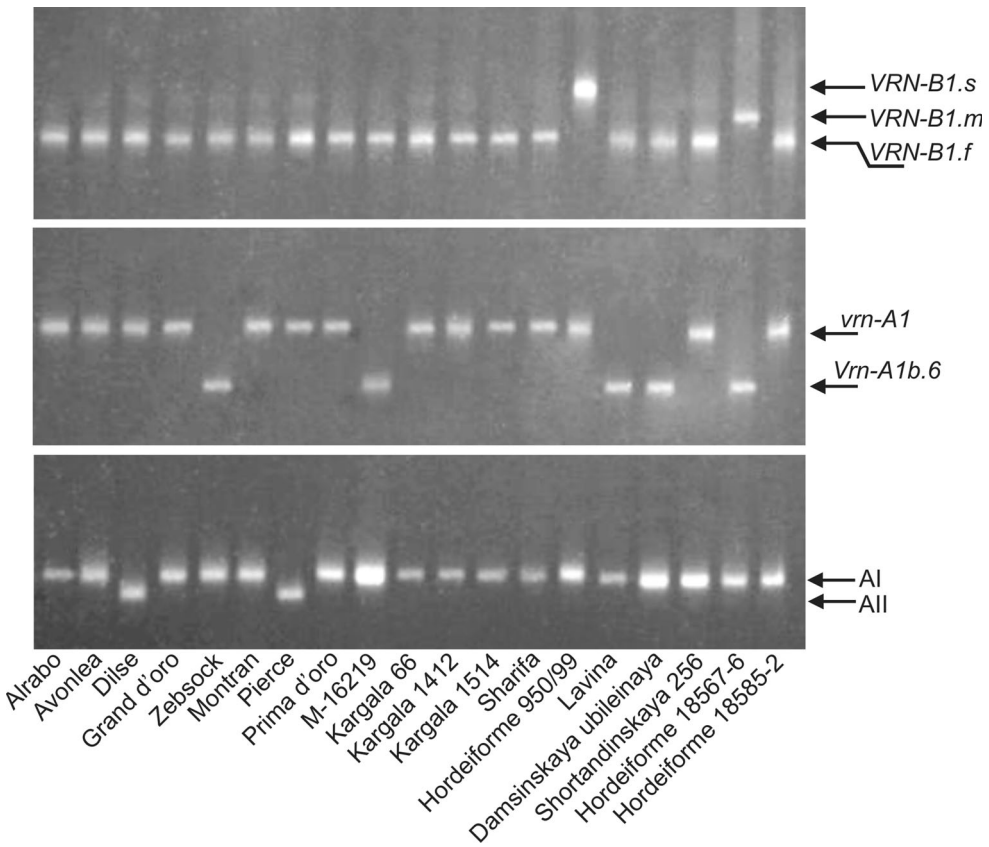


Fig. 1 Electrophoresis of the *VRN-A1, VRN-B1*, and *PPD-A1* promoter region PCR fragments (three PCR reaction mix per a gel lane). *Arrows* indicate amplicons corresponding to the *VRN1* alleles and the *Ppd-A1b* haplogroup (AI and AII). Some PCR fragments of *Vrn-A1b.6* and the amplicon of *VRN-B1.m* were sequenced and deposited in GenBank under accessions: KX139169–KX139170 and KX139168

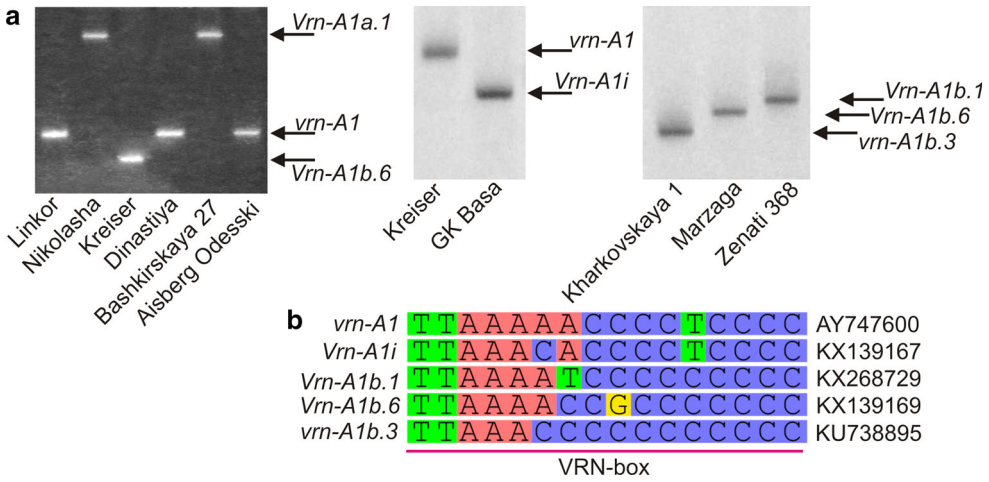


Fig. 2 Allelic variation at the *VRN-A1* gene, caused by polymorphism in the promoter region, revealed in varieties of *T. durum*. **a** PAGE of the *VRN-A1* promoter region amplicons, obtained during PCR with primers VRN1AF and VRN1-INT1R. **b** Sequence variation of the VRN-box among different *VRN-A1* alleles identified in the present study. Allele name, VRN-box sequence, and Genbank accession number are indicated

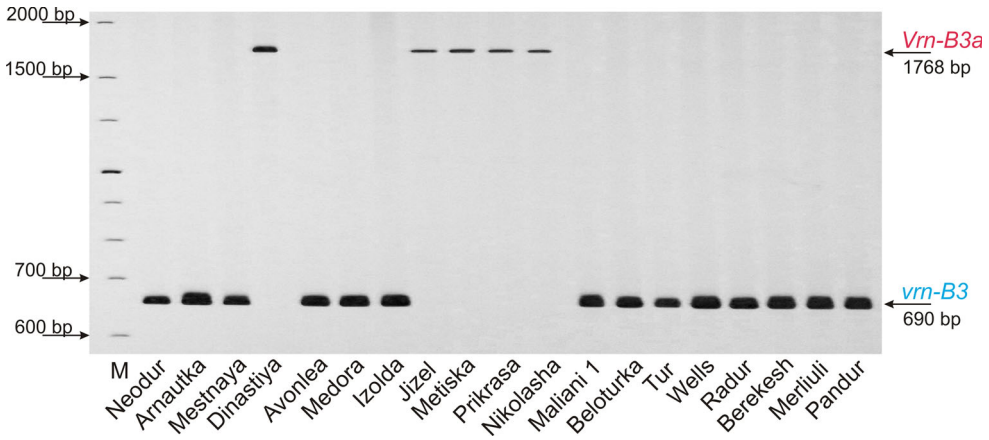


Fig. 3 Electrophoregram of the *VRN-B3* promoter region PCR fragments in PAA gel. Amplicons of the dominant *Vrn-B3a* and recessive *vrn-B3* alleles, obtained during multiplex PCR with primers FT-B-INS-F, VRN4-B-NOINS-F2, and VRN4-B-NOINS-R, are indicated. Lane M,DNA molecular size marker. A PCR fragment of *Vrn-B3a* was sequenced and deposited in GenBank under accession number KX139171

PAGE of the PCR fragments obtained using Pr1 and Pr2 pair primers was performed to distinguish of the *VRN-B1* promoter sequence variants (Fig. 1). Results demonstrated that 13 varieties of *T. durum* carry the *VRN-B1.s* allele, containing 7, 3 and 2 bp deletions, and the variety Hordeiforme 18567-6 from Kazakhstan has *VRN-B1.m* with 2 and 3 bp deletions (GenBank: KX139168), while other cultivars are characterized by the intact *VRN-B1* promoter (*VRN-B1.f* allele). Varieties with *VRN-B1.s* predominated in Russia. Investigation of the *VRN-B1* first intron by multiplex PCR using primers Ex1/C/F, Intr1/B/R3, and Intr1/B/R4 revealed that the dominant alleles of *VRN-B1*, carrying large deletions within intron-1, were predominant in Ukrainian, Russian, and Kazakhstan varieties (96 %); herewith, for these cultivars, the exceptionally *Vrn-B1c* (GenBank: KX139173) was identified. This allele also was found in single variety from Turkey (Firat 1) and Canada (Medora). The cultivar of Avonlea from USA alone carries *Vrn-B1a*. PCR fragments from Hordeiforme 18567-6 migrated through PAA gels slightly slower than amplicons of *vrn-B1*. However, this difference was not observed under electrophoresis in agarose gels. The difference between these two amplicons is likely to be caused by polymorphism within A-tracts, explaining the change of migration rate in PAA gels.

To distinguish the allelic variants of the *VRN-B3* gene, the multiplex PCR using the primers FT-B-INS-F, VRN4-B-NOINS-F2, and VRN4-B-NOINS-R was carried out (Fig. 3). In this PCR, fragments migrating at 690 bp indicated the recessive *vrn-B3*, while 1768 bp fragments detected the dominant *Vrn-B3a* allele, containing the ~5.3 kb retrotransposon insertion in the promoter region. Dominant *VRN-B3* alleles are very rare in wheat, so it was surprising that in 28 varieties of *T. durum,* the *Vrn-B3a* (GenBank: KX139171) was identified (Fig. 3). Among them, the varieties from Ukraine (79 %) predominated; five varieties were from Russia and only one from Kazakhstan. *Vrn-B3a* was revealed only in combination with dominant alleles of *VRN-A1* (25 %) alone or often with both *VRN1* genes (75 %).

The hierarchical cluster analysis results are represented in detail in Fig. 4 and are summarized in Table 2. The

cladogram from Fig. 4 shows the cluster distribution of varieties of *T. durum* depending on the content of *VRN* alleles. Additional information about the number of varieties carrying the *VRN* combination was formed from alleles of previous orders of the given trend, and the percent of varieties carrying this combination relative to all varieties possessing the allele of the current node is indicated for each node. For example, the dominant *Vrn-B3a* allele is presented in six combinations; herewith, 67.9 % varieties carrying this allele have the combination of *Vrn-A1c/Vrn-B1c/VRN-B1.f/Vrn-B3a*, i.e., characterized by the dominant alleles at the all three *VRN* genes simultaneously. The nodes of the last order indicate the number of varieties with the given combination of the *VRN* alleles and their percent in the subset of the *T. durum* varieties from a given geographical area (country). A total of 22 combinations of *VRN* alleles were identified. The most common combinations of *VRN* for varieties of *T. durum* independently of eco-geographical growing area are the *Vrn-A1c/VRN-B1.f/vrn-B1/vrn-B3* and *Vrn-A1b.6/VRN-B1.f/vrn-B1/vrn-B3* combinations, i.e., predominant varieties containing the dominant *Vrn-A1c* or *Vrn-A1b* allele alone. Three *VRN* combinations were common for the *T. durum* varieties from Ukraine: (1) all 3 *VRN* are dominant, (2) variety with *vrn-A1b.3,* and (3) varieties with winter growth habit (all 3 *VRN* are recessive). Although four combinations of *VRN* were specific for varieties from Russia all of them contained the dominant *Vrn-A1a* allele, which is not found in *T. durum* from other geographical areas.

Multiplex PCR with primers Ppd-A1proF, durum_Ag5del_F2, and durum_Ag5del_R2 was carried out on varieties of *T. durum* to identify the *PPD-A1* allelic variants and haplogroups (Fig. 1). Results showed that the great majority of varieties carry an intact promoter of *PPD-A1* (*Ppd-A1b* allele) with the AI haplogroup predominant, and the AII haplogroup contains only five varieties. The photoperiod insensitive *Ppd-A1a.2* (GenBank: KJ767781) and *Ppd-A1a.3* alleles were identified for the Georgian variety Merliuli and variety Metiska from Ukraine, respectively.

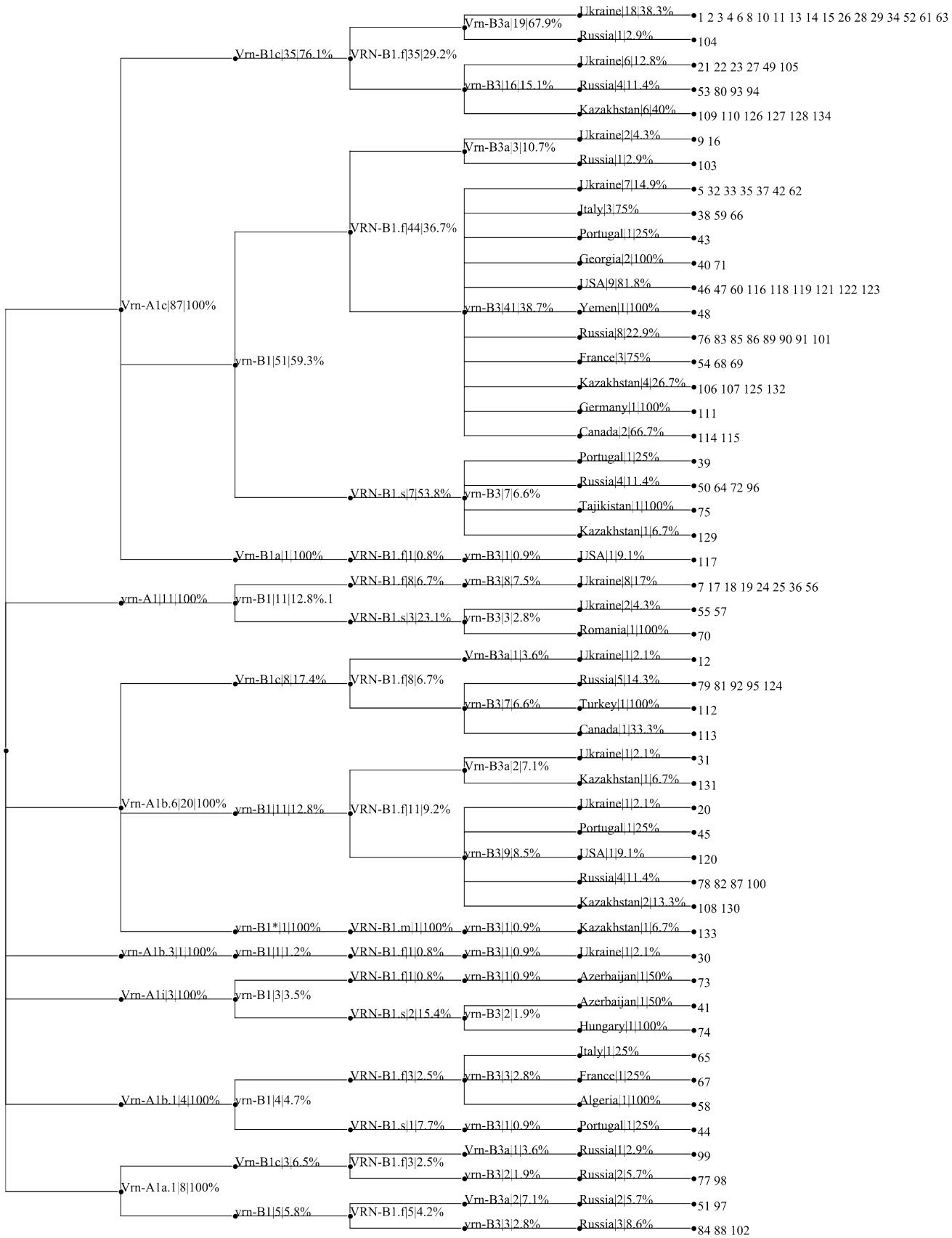
Discussion

Although durum wheat is ranked second in the area sown worldwide after bread wheat (*T. aestivum*), the varieties of *T. durum* remain poorly studied with regard to the DNA markers of the *VRN* and *PPD1* genes. In the present study, allelic variation at the *VRN-A1*, *VRN-B1*, *VRN-B3,* and *PPD-A1* genes was investigated in a collection of 134 *T. durum* varieties, representing 17 countries (Table S1). We compared allelic variation at the *VRN* genes to determine the specific compositions of these alleles in varieties of *T. durum* originating from different centers of wheat cultivation. During the investigation, the varieties of *T. durum* were analyzed using diagnostic DNA markers for the *VRN-A1*, *VRN-B1*, and *VRN-B3* promoter regions, and *VRN1* intron-1 alleles, and further grouped according to their supposed earliness through sequential clustering in an order, reflecting the influence of *VRN* genes on flowering time: *VRN-A1* > *VRN-B1* > *VRN-B3*. In this clustering, the last order (top tier) is represented by the geographical region (country), where the given combination of the *VRN* genes was found.

All spring varieties carry a dominant allele of *VRN-A1*; herewith, varieties with the *Vrn-A1c* allele (containing a large deletion in the first intron) are significantly predominant over accessions carrying mutations within the promoter region of this gene. This is not typical for other tetraploid wheat species, such as *Triticum dicoccoides*, *Triticum dicoccum*, *Triticum turgidum,* or *Triticum carthlicum*, although it is consistent with findings for *Triticum polonicum* (Muterko et al. 2016). Five *VRN-A1* allelic variants carrying mutations within the promoter region were identified. Among them, the dominant *Vrn-A1b.1* and *Vrn-A1b.6* alleles, which are widespread in tetraploid wheat species, occurred most frequently, while the *Vrn-A1i* and recessive *vrn-A1b.3* alleles that were frequently recorded in *T. turgidum* (Muterko et al. 2016), in the present study, were identified in just three and one varieties of *T. durum*, respectively.

The dominant *Vrn-A1a* allele, containing an MITE insertion within the promoter region, is more common in spring cultivars of hexaploid wheat *T. aestivum* and became widespread in cultivars after 1970. For this reason, it was supposed that *Vrn-A1a* is characteristic for hexaploid wheat but not for tetraploid wheat (Yan et al. 2004a). In our previous study, several variants of this allele were found in the A-genome of polyploid wheat. While *Vrn-A1a.2* allele was represented only in hexaploid wheat, the *Vrn-A1a.1* allele was identified also in tetraploid wheat of *T. dicoccum* (Muterko et al. 2016). In the present study, the dominant *Vrn-A1a.1* allele was identified in numerous *T. durum* varieties (20 % of all accessions with a mutant promoter of *VRN-A1*) from Russia but was not found in varieties from any others countries. This indicates the specificity of the *Vrn-A1a.1* for Russian varieties of *T. durum*.

In our previous study of the 21 *T. durum* accessions from countries outside of Russia, Ukraine, and Kazakhstan, only one accession contained the dominant *Vrn-B1a* allele (Muterko et al. 2016). Thus, on first glance, it seems that the dominant *Vrn-B1* alleles are not common in *T. durum.* However, a high frequency of *Vrn-B1c* was recorded for varieties from Ukraine, Russia, and Kazakhstan that sharply distinguish them from the *T. durum* varieties from other countries (this study and Muterko et al. 2016). Compared


Ukraine|18|38.3%
1 2 3 4 6 8 10 11 13 14 15 26 28 29 34 52 61 63
Vrn-B3a|19|67.9%
Russia|1|2.9%
104
Vrn-B1c|35|76.1%
VRN-B1.f|35|29.2%
Ukraine|6|12.8%
21 22 23 27 49 105
vrn-B3|16|15.1%
Russia|4|11.4%
53 80 93 94
Kazakhstan|6|40%
109 110 126 127 128 134
Ukraine|2|4.3%
9 16
Vrn-B3a|3|10.7%
Russia|1|2.9%
103
Ukraine|7|14.9%
5 32 33 35 37 42 62
Italy|3|75%
38 59 66
VRN-B1.f|44|36.7%
Portugal|1|25%
43
Georgia|2|100%
40 71
USA|9|81.8%
46 47 60 116 118 119 121 122 123
Vrn-A1c|87|100%
vrn-B3|41|38.7%
Yemen|1|100%
48
Russia|8|22.9%
76 83 85 86 89 90 91 101
vrn-B1|51|59.3%
France|3|75%
54 68 69
Kazakhstan|4|26.7%
106 107 125 132
Germany|1|100%
111
Canada|2|66.7%
114 115
Portugal|1|25%
39
Russia|4|11.4%
50 64 72 96
VRN-B1.s|7|53.8%
vrn-B3|7|6.6%
Tajikistan|1|100%
75
Kazakhstan|1|6.7%
129
Vrn-B1a|1|100%
VRN-B1.f|1|0.8%
vrn-B3|1|0.9%
USA|1|9.1%
117
VRN-B1.f|8|6.7%
vrn-B3|8|7.5%
Ukraine|8|17%
7 17 18 19 24 25 36 56
vrn-A1|11|100%
vrn-B1|11|12.8%.1
Ukraine|2|4.3%
55 57
VRN-B1.s|3|23.1%
vrn-B3|3|2.8%
Romania|1|100%
70
Vrn-B3a|1|3.6%
Ukraine|1|2.1%
12
Vrn-B1c|8|17.4%
VRN-B1.f|8|6.7%
Russia|5|14.3%
79 81 92 95 124
vrn-B3|7|6.6%
Turkey|1|100%
112
Canada|1|33.3%
113
Ukraine|1|2.1%
31
Vrn-B3a|2|7.1%
Kazakhstan|1|6.7%
131
Vrn-A1b.6|20|100%
vrn-B1|11|12.8%
VRN-B1.f|11|9.2%
Ukraine|1|2.1%
20
Portugal|1|25%
45
vrn-B3|9|8.5%
USA|1|9.1%
120
Russia|4|11.4%
78 82 87 100
Kazakhstan|2|13.3%
108 130
vrn-B1*|1|100%
VRN-B1.m|1|100%
vrn-B3|1|0.9%
Kazakhstan|1|6.7%
133
vrn-A1b.3|1|100%
vrn-B1|1|1.2%
VRN-B1.f|1|0.8%
vrn-B3|1|0.9%
Ukraine|1|2.1%
30
VRN-B1.f|1|0.8%
vrn-B3|1|0.9%
Azerbaijan|1|50%
73
Vrn-A1i|3|100%
vrn-B1|3|3.5%
Azerbaijan|1|50%
41
VRN-B1.s|2|15.4%
vrn-B3|2|1.9%
Hungary|1|100%
74
Italy|1|25%
65
VRN-B1.f|3|2.5%
vrn-B3|3|2.8%
France|1|25%
67
Vrn-A1b.1|4|100%
vrn-B1|4|4.7%
Algeria|1|100%
58
VRN-B1.s|1|7.7%
vrn-B3|1|0.9%
Portugal|1|25%
44
Vrn-B3a|1|3.6%
Russia|1|2.9%
99
Vrn-B1c|3|6.5%
VRN-B1.f|3|2.5%
vrn-B3|2|1.9%
Russia|2|5.7%
77 98
Vrn-A1a.1|8|100%
Vrn-B3a|2|7.1%
Russia|2|5.7%
51 97
vrn-B1|5|5.8%
VRN-B1.f|5|4.2%
vrn-B3|3|2.8%
Russia|3|8.6%
84 88 102

◀**Fig. 4** Rectangular cladogram, representing the result of controlled sequential hierarchical clustering of the *T. durum* varieties by *VRN* genes in an order reflecting their effect on flowering time (*VRN-A1* > *VRN-B1* > *VRN-B3*). For each nodes, the *VRN* allelic variants, number of varieties carrying the combination of the *VRN* alleles, which was formed from nodes of previous orders of given trend and ratio (percent) of these varieties to subset of varieties containing this node value are separated by "|". Nodes of the last tier indicate index of the *T. durum* varieties from Table S1

to other tetraploid wheat species, *Vrn-B1c* is frequent in *T. dicoccoides* and present partly in *T. dicoccum* (in contrast to *Vrn-A1c*) but was not found in *T. polonicum*, *T. turgidum*, or *T. carthlicum* (Muterko et al. 2016). Distribution of *Vrn-B1c* among the *T. durum* varieties is similar to that obtained for cultivars of bread wheat (*T. aestivum*), where *Vrn-B1c* was frequent in East Europe and Russia (Milec et al. 2013). This distribution results from previous selections and likely indicates a selective advantage of the *Vrn-B1c* allele for these geographical areas.

During the investigation of the *VRN-B1* promoter region, we found 13 varieties of *T. durum* carrying the *VRN-B1.s* allele that has been previously shown to be associated with early flowering of spring wheat accessions (Muterko et al. 2016). Furthermore, the variety of Hordeiforme 18567-6 from Kazakhstan carries the *VRN-B1.m* allele, which is less frequent in wheat and previously identified only in *T. dicoccoides* (Muterko et al. 2016).

The *Vrn-A1i* and *VRN-B1.s* alleles alone have a relatively weak effect on the vernalization response of wheat (Muterko et al. 2016). The Hungarian variety of GK Basa carrying *Vrn-A1i* and *VRN-B1.s* was grown in the glasshouse under a long photoperiod (24 h day length) and without vernalization. The heading date of this variety was 3 days later than for the accession of hexaploid wheat (PI 186391) with *Vrn-D1a* allele (*Vrn-D1a/VRN-B1.f*, facultative growth habit), namely, 145 and 142 days, respectively. This confirms our previous results for the *Vrn-A1i/VRN-B1.s* genotype (Muterko et al. 2016). However, it should be noted that planting of wheat in this way (under a 24 h photoperiod) showed corresponding changes in phases of development (steam elongation and flowering time) with an approximate 70 days delay. This later flowering phenotype is a precedent among *T. durum* varieties with a facultative growth habit, and opens new opportunities for breeding of durum wheat cultivars. However, as previously was noted (Muterko et al. 2016), the genetic analysis will be required to confirm the observed association between mutations within the *Vrn-*

Table 2 Distribution of *VRN* allelic combinations among varieties of *T. durum* from different geographical areas

VRN alleles combination	Number of varieties	Frequency (%)					
		America	Europe	Russia	Ukraine	Kazakhstan	Other
Vrn-A1c/VRN-B1.f/Vrn-B1c/Vrn-B3a	19			5	95		
Vrn-A1c/VRN-B1.f/vrn-B1/vrn-B3	41	27	18	20	17	10	7
vrn-A1/VRN-B1.f/vrn-B1/vrn-B3	8				100		
Vrn-A1c/VRN-B1.f/vrn-B1/Vrn-B3a	3			33	67		
Vrn-A1b.6/VRN-B1.f/Vrn-B1c/Vrn-B3a	1				100		
Vrn-A1b.6/VRN-B1.f/vrn-B1/vrn-B3	9	11	11	45	11	22	
Vrn-A1c/VRN-B1.f/Vrn-B1c/vrn-B3	16			26	37	37	
vrn-A1b.3/VRN-B1.f/vrn-B1/vrn-B3	1				100		
Vrn-A1b.6/VRN-B1.f/vrn-B1/Vrn-B3a	2				50	50	
Vrn-A1c/VRN-B1.s/vrn-B1/vrn-B3	7		14	58		14	14
Vrn-A1i/VRN-B1.s/vrn-B1/vrn-B3	2		50				50
Vrn-A1b.1/VRN-B1.s/vrn-B1/vrn-B3	1		100				
Vrn-A1a.1/VRN-B1.f/vrn-B1/Vrn-B3a	2			100			
vrn-A1/VRN-B1.s/vrn-B1/vrn-B3	3		33		67		
Vrn-A1b.1/VRN-B1.f/vrn-B1/vrn-B3	3		67				33
Vrn-A1i/VRN-B1.f/vrn-B1/vrn-B3	1						100
Vrn-A1a.1/VRN-B1.f/Vrn-B1c/vrn-B3	2			100			
Vrn-A1b.6/VRN-B1.f/Vrn-B1c/vrn-B3	7	14		72			14
Vrn-A1a.1/VRN-B1.f/vrn-B1/vrn-B3	3			100			
Vrn-A1a.1/VRN-B1.f/Vrn-B1c/Vrn-B3a	1			100			
Vrn-A1c/VRN-B1.f/Vrn-B1a/vrn-B3	1	100					
Vrn-A1b.6/VRN-B1.m/vrn-B1/vrn-B3	1					100	

A1i and *VRN-B1* promoter regions, vernalization response, and flowering time.

Previous studies have revealed no less than four allelic variants of *VRN-B3*—two dominant and two recessive (Yan et al. 2006; Chen et al. 2013; Derakhshan et al. 2013). Furthermore, it has been shown that dominant alleles of this gene are less frequent in wheat and in particularly in varieties of *T. aestivum* (Yan et al. 2006; Zhang et al. 2008; Iqbal et al. 2011; Derakhshan et al. 2013; Chen et al. 2013). In the present study, *Vrn-B3a* was found in 28 varieties of *T. durum*. Interestingly, almost all these varieties were from Ukraine. This allows us to assume that currently, the pool of *T. durum* varieties from Ukraine is the largest genetic source of the dominant *Vrn-B3a* allele in wheat. In addition, our data indicate that in contrast to the currently popular view, the dominant *Vrn-B3a* allele has an earlier origin in tetraploid wheat, but not hexaploid wheat.

Overall, the 22 allelic combinations at the *VRN* genes were identified. From them, three were described for varieties from Ukraine and four for Russian. Furthermore, the distinctive features of Ukrainian and Russian varieties include a high frequency of the *Vrn-B1c* allele and presence of genotypes with both dominant *VRN1* genes. The most of Ukrainian varieties carry of the dominant *VRN-A1, VRN-B1*, and *VRN-B3* alleles simultaneously. As is already known, allele frequency is an indicator of the past selection in breeding. For this reason, it is likely that the presence of three dominant *VRN* alleles in Ukrainian varieties of *T. durum*, leading to the early flowering, is an important regional adaptation, which allows it to avoid the effects of high summer temperatures and phytopathogen infection.

The analysis of the *PPD-A1* allelic variants herein found that the PI *Ppd-A1a* alleles are not more frequent in *T. durum* varieties, while for varieties of hexaploid wheat *T. aestivum* from these geographical regions, the photoperiod insensitive alleles (in particularly *Ppd-D1a*) were permanently introduced during breeding as an agronomically valuable adaptation in the days of the "green revolution". Almost all varieties have the AI haplogroup of *Ppd-A1b* that is frequent in *T. durum* (Muterko et al. 2015b). The AII haplogroup of *Ppd-A1b* is found only for varieties from USA, Romania, and Russia that is also consistent with the trend of distribution of the *PPD-A1* haplogroups among different eco-geographic areas (Muterko et al. 2015b). These data indicate that likely none of these haplogroups had been selected in the breeding of varieties of *T. durum*. In our previous study of 6 tetraploid wheat species, including *T. dicoccoides*, *T. dicoccum*, *T. carthlicum*, *T. turgidum*, *T. polonicum*, and *T. durum*, the PI *Ppd-A1a* alleles were not identified, thus reaffirming their later emergence in cultivars of *T. durum*. However, their low frequency, revealed in the present study, indicates a more recent timing of this event, explaining the limited distribution of PI *Ppd-A1a* alleles among varieties of durum wheat.

Conclusions

Here, we demonstrate that the American and European varieties of *T. durum* have a similar genotype at the *VRN* genes, while varieties from Ukraine, Russia, and Kazakhstan are characterized by the specific allelic composition of these genes. In particular, for cultivars from these countries, the most commonly held condition is the presence of dominant alleles for both *VRN1* genes simultaneously. Furthermore, Ukrainian varieties provide the largest natural genetic source of the dominant *Vrn-B3a* allele, and, hence, among them predominant varieties with carrying dominant alleles of three *VRN* genes simultaneously (*VRN-A1*, *VRN-B1*, and *VRN-B3*). The specificity of Russian varieties can be attributed to the high frequency of the *Vrn-A1a.1* allele. In both the cases, the *Vrn-B3a* and *Vrn-A1a.1* alleles were previously identified only in hexaploid wheat, and in the present study, for the first time, the high frequency of these alleles in varieties of tetraploid *T. durum* wheat was revealed. Similarly, for certain varieties of *T. durum*, the photoperiod insensitive *Ppd-A1a* alleles were identified for the first time.

Author contribution statement AM carried out the experiments, developed an algorithm of the controlled sequential hierarchical clustering, sequenced of the *VRN-A1*, *VRN-B1*, and *Vrn-B3a* alleles, and wrote the manuscript. RK cloned and sequenced the *Vrn-A1i* and *Vrn-A1b.6* alleles and contributed to the preparation of the manuscript. EA revised the manuscript, contributed to the discussion, and participated in preparing the manuscript. All authors read and approved the final manuscript.

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