Allelic Variations within Vrn-Genes at Different Ploidy Levels

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Rapid climate changes, with higher warming rates during winter and spring seasons, dramatically affect the vernalization requirements, one of the most critical processes for the induction of wheat reproductive growth, with severe consequences on flowering time, grain filling, and grain yield. Specifically, the *Vrn* genes play a major role in the transition from vegetative to reproductive growth in wheat. Recent advances in wheat genomics have significantly improved the understanding of the molecular mechanisms of *Vrn* genes (*Vrn-1*, *Vrn-2*, *Vrn-3*, and *Vrn-4*), unveiling a diverse array of natural allelic variations.

ploidy wheat durum wheat allelic variations

1. Allelic Variation of Vrn-1 at the Promoter Level

Genetic variations at the promoter level may significantly impact *Vrn-A1* expression and regulation [1]. Thus, understanding their diversity in genotypes with different ploidy levels can provide a valuable resource to further investigate the genetic basis of FT regulation in wheat. The promoter of *Vrn-1* is considered a repertoire of regulatory elements, of which CArG-box, VRN-box, and ACGT-motif are the most studied [1]. VRN-box is characterized by a 16 bp region (“TTAAAAACCCCTCCCC”) and is considered the most influential on the “winter-spring” growth habit [2], whereas CArG-box (a common binding site for MADS-box) is not critical, since genotypes with a fully deleted CArG-box region show a preserved vernalization machinery [3][4]. Distinct novel genetic variations have been revealed to be situated within the regulatory region of *Vrn-A1*.

Vrn-A1a stands out as one of the most significant and potent spring alleles [5]. It has a duplicated promoter region carrying characteristic foldback elements. The two fragments differed from the recessive *vrn-A1* allele by the insertion of a 222 bp foldback element in the larger fragment and a 131 bp foldback element in the smaller one [5]; it was reported that *Vrn-A1a* was predominant in spring varieties released in the United States and Argentina between 1970 and 2004 and hypothesized that the increase in *Vrn-A1a* frequency in this germplasm was related to the introduction of the semi-dwarf germplasm from CIMMYT during the 1970s. Indeed, the allele *Vrn-A1a* was not present in a collection of durum wheat landraces analyzed by Royo et al. [6], which were typically characterized by tall plants, long coleoptiles, and early vigor. Later, Muterko et al. [3] described three different variants of *Vrn-A1a* designated as *Vrn-A1a.1*, *Vrn-A1a.2*, and *Vrn-A1a.3*. *Vrn-A1a.1* and *Vrn-A1a.3* corresponded to the known *Vrn-A1a* allele described by Yan et al. [5] in hexaploid and tetraploid wheat, whereas *Vrn-A1a.2* was novel and compared to *Vrn-A1a* was characterized by two deletions (16 bp and 4 bp) within the MITE element [3][5][7].

Tranquilli and Dubcovsky [8] also identified variants within the VRN-box. *vrn-*A^m*1* and *Vrn-*A^m*2* were found in diploid *T. monococcum* and were reported as dominant for spring and winter growth habits. Sequence analysis revealed SNPs in the A-tract of the VRN-box in *T. turgidum* and *T. durum* and validated the identification of the *vrn-*A^m*1* allele for the accession of *T. monococcum* [9]. Subsequently, Muterko et al. [9] demonstrated the existence of a 10 bp deletion in diploid wheat (*T. monococcum*), as well as some natural variants, *Vrn-*A^m*1a*, *vrn-*A^m*1b* [4], and *Vrn-*A^m*1g* [10], that exhibited deletions or a complete absence of the CArG-box, as in the specific case of *vrn-*A^m*1b*. Natural variants within the other regulatory regions (i.e., CarG box and/or G box) were also identified in tetraploid and hexaploid wheat [3][10][11]. Two alleles, named *Vrn-A1d* and *Vrn-A1e*, harbored 32 bp and 54 bp deletions within the CarG box, respectively [5], whereas *Vrn-A1f* exhibited a substantial 50 bp deletion within the −62 and −112 bp region; it also displayed a smaller 8 bp deletion within the G box [11] as well as a polymorphism within the A-tract (A replaced by G) [9]. This allele was first described by Golovnina et al. [11] in a collection of wild diploids (*T. boeoticum* and *T. urartu*) and tetraploid (*T. araraticum* and *T. timopheevii*) wheat. In addition to *Vrn-A1f*, Golovnina et al. [11] described two other variants called *Vrn-A1g* and *Vrn-A1h* as having 34 bp and 20 bp deletions near the CArG- box, respectively, in addition to the minor deletion of 8 bp in the G box. Among them, the dominant *Vrn-A1g* allele was reported as extremely rare in both *T. monococcum* and *T. boeoticum* [11]. Ivaničová et al. [12] designed a *Vrn-A1f-like* allele from *T. militinae* (Zhuk. and Migush.) (2n = 4x = 28, AtGG genome), a wild wheat that originated from a hybridization event separate from emmer wheat and belongs to the *T. timopheevii* (Zhuk.) group. Comparison between *Vrn-A1f-like* and *Vrn-A1a* revealed major mutations in the promoter region [the nonexistence of the Spring fold element (SFE) insertion and two deletions (8 base pairs and 50 base pairs) positioned downstream of the CArG box] but also within the first intron [12]. In spring *T. dicoccum*, a dominant allele known as *Vrn-A1k*, characterized by a 42 bp insertion at −108 bp, was reported by Muterko and Salina [13], whereas *Vrn-A1j* was described in *T. compactum* as carrying a deletion of 54 bp between −140 and −87 in the promoter [14].

2. Allelic Variation of Vrn-1 at Gene Body Level

Regarding the allelic variation at the gene body level, *Vrn-A1c* [7] and *Vrn-A1L* [15] alleles were discovered in tetraploid wheat, which were characterized by 5.5 kb and 7.2 kb deletions in the first intron, respectively [15]. Compared to the recessive *vrn-A1* allele, *Vrn-A1c* in hexaploid wheat had eight unique SNPs and five unique 1 bp indels in the first intron [7]. Additionally, an allele called *Vrn-A1ins* was identified, which possesses a 0.5 kb insertion within intron 1 of the diploid *T. monococcum* [15]. Furthermore, the *vrn-A1u* allele was observed, and was characterized by a 1.4 kb deletion within intron 1 of *T. urartu* and polyploid species with an A-genome [15]. Sehgal et al. [16] and Steinfort et al. [17] described the *Vrn-A1f* and *VRN-A1AUS28709 Ai2* alleles in *T. aestivum*, respectively, harboring a deletion in intron 1. Furthermore, *T. araraticum* and *T. timopheevii* as the tetraploid species of the *Timopheevi* group are characterized by *Vrn-A1f-del* (2.7 kb deletion at intron 1 in *T. araraticum), Vrn-A1f-ins* (0.4 kb insertion at intron 1 in *T. timopheevii*), and *Vrn-A1f-del/ins* (0.4 kb insertion and 2.7 kb deletion at intron 1 in *T. timopheevii*), plus the deletions and the polymorphism in the promoter as described for allele *Vrn-A1f* [18], while *T. militinae* possesses an MITE transposon (0.4 kb insertion) and a 2.7 kb deletion in intron 1, and also exhibits a host duplication of nine base pairs in the first intron, and two synonymous SNPs in exon 7 and exon 8 [12]. Intriguingly, a polymorphism in the coding sequence of the recessive allele has been exclusively identified for *Vrn-A1* [19][20].

Based on the presence of “C → T” transition within exon 4 at position 20 bp of *Vrn-A1*, two different haplotypes were initially distinguished (Ex4C, wild type and Ex4T, mutant type). Similarly, the same transition (“C → T”) which led to the substitution of alanine for valine (Ala180/Val180) within exon 7 was observed [19]. Muterko and Salina [14] reported then a survey of exon 4 haplotypes in 12 tetraploid and hexaploid wheat species. The authors found that the Ex4T haplotype was present only in the hexaploid wheat *vrn-A1* allele, and exclusively in combination with the Ex4C haplotype in accessions of hexaploid wheat carrying *Vrn-A1* multi-copies. In addition, to denote the *Vrn-A1* exon 4 haplotype, Muterko and Salina used the previously available nomenclature [13], further expanding it. Using the abovementioned nomenclature, mutations within intron-4 were used to distinguish four haplotypes (Ex4C.s, Ex4C.m, Ex4C.f, and Ex4C.sph) [13]. The first three were named based on their migration velocity (s: slow, m: middle, f: fast), whereas Ex4C.sph was detected only in *T. sphaerococcum*. Furthermore, Muterko and Salina [14] identified two polymorphisms in exon 4 and exon 7 on the *Vrn-A1j* (exon 7) and *Vrn-A1k* (both exon 4 and 7) alleles.

The dominant alleles of the *Vrn-B1* and *Vrn-D1* loci exhibit variations from the recessive alleles, mainly characterized by insertions or deletions within the first intron [2][7][21]. The allele *Vrn-B1a*, identified in 2005 by Fu and colleagues [7], was characterized by a 6850 bp deletion in intron 1, whereas a similar allele called *Vrn-B1b* (the same 6850 bp deletion of *Vrn-B1a* plus a 36 bp indel) was described by Santra et al. [22]. *Vrn-B1c*, discovered by Chu et al. [23] and later by Milec et al. [24], differs from the others by an 817 bp deletion and 432 bp duplication in intron 1. Zhang et al. [25] reported a novel dominant allele, *Vrn-B1d*, in the Chinese spring Hongchunmai. The allele contained several genetic divergences within intron 1 compared to *vrn-B1*, including a large 6850 bp deletion (670–7519 bp), one small 187 bp deletion (7851–8037 bp), an SNP (T/C at 7845 bp), and one 4 bp mutation (TTTT to ACAA, 7847–7850 bp). In 2021, Strejčková and colleagues [26] found a novel allele called *Vrn-B1f*, which was characterized by an 836 bp insertion within intron 1 in bread wheat.

3. Copy Number Variations of Vrn-1

Copy number variation (CNV) can also greatly impact *Vrn-1* gene function [27], thus influencing wheat adaptation and flowering time [27][28][29]. In bread wheat, CNV in recessive and dominant *Vrn-1* alleles has been reported [27][29][30]. A different number of copies of *Vrn-A1* led to different vernalization requirements among winter wheat cultivars [27][28]. The heading date of winter wheat was affected by allelic variation associated with CNV at the *Vrn-A1* locus [31]. The earlier flowering after a short vernalization period relates to a low copy number at *Vrn-A1* [27]. In other words, the CNV of the *Vrn-A1* gene strongly impacts vernalization requirements and late flowering [27]. Zhu et al. [28] recommended that choosing wheat varieties with three copies of the recessive *vrn-A1* gene would be a viable method to increase the frost tolerance ability of wheat because of the association between increased *Vrn-A1* copy number and greater frost tolerance.

More than 90% of winter varieties of *T. aestivum* carry two to three copies of the *Vrn-A1* gene [29]. Muterko and Salina [30] represented the copy number of *Vrn-A1* with the alternative exon 4 haplotype in spring and winter accessions of tetraploid and hexaploid wheat. Another study reported the duplication of *Vrn-A1b.3* in *T. dicoccum* and the *Vrn-A1b.3* and *Vrn-A1b.2* in hexaploid *T. spelta* [32]. Muterko [32] described that duplicated *Vrn-A1b.2* was related to the awnless spikes in *T. spelta*, whereas Würschum et al. [29] found that the geographical patterns of *Vrn-A1* copy number variations were compatible with their roles in promoting wheat's worldwide adaptability.

CNV at the *Vrn-B1* locus was also reported by Muterko and Salina [30] in *T. compactum* (Host) and *T. spelta* (L.), although Strejčková et al. [26] reported that *Vrn-B1* and *Vrn-D1* exist in a single copy. By contrast, the authors found that recessive *Vrn-A1* has one to four copies, whereas the dominant *Vrn-A1* has one or two copies [26].

4. Allelic Variation of Vrn-1 at Different Ploidy Levels

On the AA genome, three recessive alleles (*vrn-A*^{m}*1*, *vrn-A1u*, and *vrn-A*^{m}*1b*) have been identified in diploid species [10][11][15][33].

The *vrn-A*^{m}*1* allele was found in all diploid species, and to date, it represents the only variant reported in *Triticum sinskajae* A. Filat. et Kurk. [10][11][15][33]. By contrast, *vrn-A1u*, identified in *T. urartu* Thum. ex Gandil by Golovnina et al. [11], is identical to the recessive *vrn-A1* reported in polyploid wheat and differs from *vrn-A*^{m}*1* for a deletion in the promoter region [11][15]. The *vrn-A*^{m}*1b* allele instead was only detected in accessions of *T. monococcum* L. [4][33]. Dominant alleles were also identified in diploid wheat (e.g., *T. monococcum*) [11]. For example, two dominant alleles (*Vrn-A*^{m}*1f* and *Vrn-A*^{m}*1a Vrn-A1h*) were found in *T. boeoticum* Boiss. and *T. monococcum* [11][15], whereas, so far, no dominant alleles have been identified in *T. urartu* [11][15].

In tetraploid species, the recessive allele *vrn-A1* was inherited from diploids, presumably from *T. urartu,* since no differences were observed at the promoter level [15][34], and to date, three recessive alleles [*vrn-A1*(*vrn-A1u*), *vrn-A1b.3*, *vrn-A1b.4*] have been described in both *Timopheevii* A. Filat. et Dorof. and *Dicoccoides* Flaksb. sections [3][9][15]. As suggested by Konopatskaia et al. [34], dominant alleles such as *Vrn-A1a.3*, *Vrn-A1e*, *Vrn-A1i* and *Vrn-A1b* might originate through deletion (*Vrn-A1b* and *Vrn-A1e*), insertion (*Vrn-A1a.3*), or substitution (*Vrn-A1i*) events from the recessive *vrn-A1.* Interestingly, dominant alleles of *Vrn-A1b* except *Vrn-A1b.7* and *Vrn-A1e* were distributed only in the dicoccoides section (AABB), suggesting that they evolved from *vrn-A1* after the section separation [34][35]. By contrast, *Vrn-A1b.7* was found in both the Emmer lines (AABB) and the *Timopheevii* lines (AAGG), suggesting that they originated from a common tetraploid ancestor [34]. Shcherban and Salina [21] reported that the presence of new dominant *Vrn-1* alleles was not related to the origin in diploids, since the allele set found in *T. dicoccoides* differs from *Timopheevii,* indicating an independent origin of dominant alleles within these two allopolyploids [21]. In *T. timopheevii* Zhuk. and *T. araraticum* Jakubz. have only one dominant allele (*Vrn-A1f*), which originated from the recessive *vrn-A*^{m}*1* of *T. monococcum*, *T. urartu*, *T. boeoticum*, and was described at the *Vrn-A1* locus [36], whereas ten dominant alleles were identified in different tetraploid wheat species of section *Dicoccoides* Flaksb. [*Vrn-A1a*(*Vrn-A1a.3*), *Vrn-A1b*(*Vrn-A1b.1*), *Vrn-A1b.2*, *Vrn-A1b.5*, *Vrn-A1b.6*, *Vrn-A1e*, *Vrn-A1f*, *Vrn-A1i*, and *Vrn-A1d*] [9][11][15][35]. *Vrn-A1a.3* was restricted to *T. dicoccum* and *T. dicoccoides,* whereas the dominant *Vrn-A1d* allele has been found in both *Timopheevii* A. Filat. et Dorof. and *Dicoccoides* Flaksb. sections and it probably arises from *Vrn-A1b* variants due to an extended deletion. Konopatskaia et al. [34] alternatively reported that the two deletions within *vrn-A1* could originate from the *Vrn-A1d* locus [34]. *Vrn-A1d* probably originated at the tetraploid level, and it was not inherited in hexaploid wheat, as suggested by Konopatskaia et al. [34], even though most of the known dominant *Vrn-1* alleles in common hexaploid wheat originated at the tetraploid stage [*Vrn-A1a.1*, *Vrn-A1a.2*, *Vrn-A1b*(*Vrn-A1b.1*), *Vrn-A1b.2*, *Vrn-A1b.6*, *Vrn-A1c*, and *Vrn-A1f*] [7][23][37][38].

In hexaploid wheat, before the identification of *vrn-A1b.3* in *T. vavilovii* (Thum.) Jakubz. and *T. spelta* L. by Muterko et al. [3][9], *vrn-A1* was the only recessive allele identified [3][5][7].

In tetraploid wheat, four dominant alleles at the *Vrn-B1* locus were described [3][9], each characterized by mutations within the promoter region (such as insertion of repeated elements or short deletions) [9][11][23][39].

Vrn-B1a is the only dominant allele identified in the *dicoccoides* section and *durum* accessions [7][9][11], whereas *Vrn-B1c* probably originated from *Vrn-B1a* due to an additional deletion of 0.8 kb and a duplication of 0.4 kb [3]. Also, the *Vrn-B1b* allele appears to have originated from *Vrn-B1a*, since along with a deletion in the first intron, it also harbors a 36 bp deletion plus an additional SNP [22]. This allele was described in common wheat originating from North America and was associated with the spring growth habit [38]. The *Vrn-B1dic* promoter differs from *vrn-B1* for 29 nucleotide substitutions, one deletion, and one SNP insertion in the region spanning −220 to −155 bp upstream of the start codon, and it was found only in a genotype belonging to *T. dicoccoides* [34].

Shcherban et al. [21] identified one accession of *T. turanicum* Jakubz. (AABB) with the *Vrn-B1a* allele that does not correspond to the dominant *Vrn-B1a* for an insertion in the promoter [11]. Interestingly, the insertion was homologous to that identified in the *Vrn-A1a* allele, although the position was different (−100 from the start codon).

The dominant *Vrn-D1a* allele was found in the near-isogenic line TDE and it abounded in spring wheat adapted to tropical and subtropical regions [40][41]. *Vrn-D1b* arises from *Vrn-D1a* due to SNP in the CArG-box region [42]. The *Vrn-D1c* allele was found in three out of 205 Chinese wheat cultivars [37]. In the same year, Muterko et al. [43] found the *Vrn-D1s* allele, which is associated with spring form. Shcherban et al. [21] reported that the distribution of spring forms along with different alleles at *Vrn-1* is largely due to artificial selection based on different climatic conditions. For example, dominant haplotypes at the *Vrn-A1* and *Vrn-B1* loci were observed in cultivars from northern and central Europe and from Russia [21], whereas the monogenic dominant haplotypes contained at either *Vrn-B1* or *Vrn-D1* were mostly widespread in cultivars for southern Europe [21][44].

5. Allelic Variation of Vrn-2, Vrn3, and Vrn4 Genes

The identification of natural variations in *Vrn-2* genes may prove difficult due to the limited characterization of the *Vrn-2* gene in hexaploid wheat. Indeed, few natural variations in the promoter and/or in the first intron of *Vrn-2* genes (*Vrn-A2*, *Vrn-B2*, *Vrn-D2*, and *Vrn-S2*) were identified and characterized. They were originally observed in diploid wheat (*T. monococcum*) [45]. Furthermore, a previous development of a tetraploid wheat line lacking functional copies of *Vrn-2* has been documented [46]. In addition, various hexaploid wheat cultivars may have undergone multiple events of duplication, deletion, and translocation involving *Vrn-B2*. Consequently, the task of identifying specific variations becomes challenging [47]. Unlike *Vrn-1*, *Vrn-3*, and *Vrn-4* genes that are dominant for spring growth habit, *Vrn-2* genes are dominant for winter growth habit [45]. *Vrn-B2* is generally functional, whereas

Vrn-A2 is non-functional in tetraploid wheat [48][49]. Tan and Yan [47] isolated *Vrn-2* from hexaploid winter wheat cultivars Jagger and 2174, reporting no differences at *Vrn-A2* or *Vrn-D2*, while two copies of *Vrn-B2* were found in 2174, indicating that Jagger carried a *null* allele. The first copy (*Vrn-B2a.1*) was 2327 bp long and had a 2087 bp insertion between the start and stop codon plus a 144 bp insertion before the start codon, and a 96 bp insertion after the stop codon, whereas *Vrn-B2a.2* had an extra 'CAC' motif at positions 136–138 from the start codon and five SNPs compared with *Vrn-B2a.1* [47]. The cloned *Vrn-D2* was 2364 bp in length, where 239 bp corresponded to an insertion before the start codon and 96 bp to an insertion after the stop codon [47]. Distelfeld et al. [49] reported *Vrn-S2* in *Ae. speltoides* and *Vrn-D2* in *Ae. tauschii,* concluding that the winter growth habit of most of the *Ae. speltoides* and *Ae. tauschii* accessions was probably due to functional *Vrn-2*. The ZCCT1 and ZCCT2 proteins from both species showed no mutations in the conserved amino acids of the CCT domains [49].

Several natural variations were also detected and characterized in the promoter and/or in the first intron of *Vrn-3* (*Vrn-A3*, *Vrn-B3*, and *Vrn-D3*). Recently, Nishimura et al. [50] reported in wild emmer wheat six *Vrn-A3* alleles with the 7- and 25 bp insertions in the promoter region, namely, *Vrn-A3a-h2*, *Vrn-A3a-h3*, *Vrn-A3a-h4*, *Vrn-A3a-h5*, *Vrn-A3a-h6*, and *Vrn-A3c-h2*. Similar insertions (i.e., *Vrn-A3a-h2* and *Vrn-A3c-h1*) were also found in cultivated tetraploid and hexaploid wheat [50]. Yan et al. [51] identified the *vrn-A*^{m}*3* allele in *T. monococcum*, which is characterized by a polymorphism in the promoter region. The *Vrn-B3* locus in tetraploid and hexaploid wheat is defined by five dominant alleles, all linked to modifications in the promoter. Yan et al. [51] identified the *Vrn-B3a* allele characterized by the insertion of 5300 bp in the promoter region. Later, Chen et al. [20] showed two novel alleles: *Vrn-B3b*, with an insertion of 890 bp in the promoter, and *Vrn-B3c*, characterized by two deletions (20 bp and a 4 bp) in the promoter of *Vrn-B3a*. Berezhnaya et al. [52] discovered two novel allelic variants of the *Vrn-B3* gene in common wheat varieties from Russia. These alleles were designated the *Vrn-B3d* and *Vrn-B3e* alleles and had 1615 bp and 160 bp insertions in the promoter, respectively [52]. Among the alleles described for *Vrn-3*, Muterko et al. [3] reported a high frequency of *Vrn-B3a* in *T. durum* varieties from Ukraine, Russia, and Kazakhstan. Finally, Bonnin et al. [53] demonstrated the presence of polymorphic sites within four haplotypes in the A genome (*TAFTAh1*, *TAFTAh2*, *TaFTAh3*, and *TAFTAh4*), whereas two were identified in the D genome (*TAFTDh1* and *TAFTDh2*), and only one line (BT21) showed a polymorphism in the B genome (*TaFTBBT21*) of *Vrn-3*. All five affected sites (three SNPs and two deletions) were found within the first intron [54]. Additionally, a single polymorphism for genome D was observed, consisting of an INDEL of one G in the third exon [54].

Vrn-4 is an early flowering allele and is comparatively less comprehended in comparison to the preceding three vernalization genes. The Australian cultivar Gabo was the first to identify *Vrn-4* [55][56], and subsequently, it was backcrossed into Triple Dirk to produce an isogenic line called TDF [56]. This locus was assigned to chromosome 5D [57] and is now recognized as *Vrn-D4* [58]. Although only the D genome has been identified thus far as having the natural variation for flowering time in the centromeric region of homologous group 5 chromosomes, the arm position of *Vrn-D4* in wheat is yet unclear [59]. The *Vrn-D4* locus might play a crucial role in the variation in flowering time in hexaploid wheat germplasm, and it seems to have undergone independent evolution from the vernalization pathway in dicot species [45].

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