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Molecular characterization of a novel vernalization allele *Vrn-B1d* and its effect on heading time in Chinese wheat (*Triticum aestivum* L.) landrace Hongchunmai

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Abstract Flowering time of wheat cultivars contributes greatly to the adaptability to environmental conditions and it is largely controlled by vernalization genes. In this study, 262 Chinese mini-core wheat cultivars were used to identify the allelic variation at *VRN-B1* locus. A novel dominant allele *Vrn-B1d* was found in Chinese spring wheat landrace cultivar Hongchunmai. This allele contained several genetic divergence within the first intron comparing to the recessive allele *vrn-B1*, including one large 6850-bp deletion (670–7519 bp), one small 187-bp deletion (7851–8037 bp), one unique SNP (T to C, 7845 bp), and one 4-bp mutation (TTTT to ACAA, 7847–7850 bp). Meanwhile, it was also different from the three known dominant alleles at *VRN-B1* locus. Two pairs of primers were designed to identify the novel allele *Vrn-B1d* and other four known alleles of *VRN-B1*. A multiplex PCR was established to discriminate all five alleles simultaneously. The greenhouse experiment with high temperature (non-vernalizing condition) and long light showed that F_2 plants containing *Vrn-B1d* allele headed significantly earlier than those with recessive *vrn-B1* allele, suggesting that *Vrn-B1d* is a dominant allele conferring the spring growth habit. This study provides a useful germplasm and molecular markers for wheat breeding.

Keywords Bread wheat · *VRN-B1* · PCR marker · Multiplex PCR · Heading date

Introduction

Flowering time influences the adaptability of wheat to a large range of environments and is mainly controlled by three types of genes, vernalization (*VRN*), photoperiod (*PPD*), and earliness per se (*ESP*) genes (Yasuda and Shimoyama 1965; Kato and Yamagata 1988; Hanocq et al. 2004). The *VRN* genes in wheat greatly contribute to flowering time and growth habits, dividing wheat into winter and spring types. To date, four *VRN* genes (*VRN1*, *VRN2*, *VRN3*, and *VRN4*) have been cloned and characterized in wheat (Yan et al. 2003, 2004b, 2006; Kippes et al. 2015).

The *VRN1* gene, a flowering promoter that has an indispensable role in the floral transition pathway of wheat (Loukoianov et al. 2005; Shitsukawa et al. 2007), encodes a MADS-box transcription factor closely related to the Arabidopsis AP1/FRUITFULL family (Yan et al. 2003; Moon et al. 2003). Vernalization treatment can up-regulate the expression of *VRN1* conferring early flowering time (Yan et al. 2003). Insertions or deletions within the first intron and the promoter of *VRN1* genes are associated with dominant alleles for

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spring growth habit (Yan et al. 2004b; Fu et al. 2005). The *VRN2* gene, a dominant repressor of flowering, encodes two linked and related proteins designated *ZCCT1* and *ZCCT2* (zinc finger-CCT domain genes), featured by the presence of a putative zinc finger and a CCT (for CONSTANS (CO), CONSTANS-LIKE (CO-Like), and TIMING OF CAB EXPRESSION1 (TOC1) domain (Yan et al. 2004b). The *VRN2* is down-regulated by vernalization treatment and *VRN1* expression. The *VRN2* is up-regulated to block flowering by repressing *FT* (*FLOWERING LOCUS T* gene) under long day condition (Trevaskis et al. 2007). Deletion and mutation of both *ZCCT1* and *ZCCT2* genes are associated with recessive alleles for spring growth habit (Fu et al. 2005; Hemming et al. 2009; Yan et al. 2004a). Due to the dominant allele of *VRN2* conferring the winter growth habit, at least one functional copy of *VRN2* combined with homozygous recessive alleles at all three *VRN1* are required to confer winter growth habit in hexaploid wheat. *VRN3* is a homologous gene of the Arabidopsis *FT* (Yan et al. 2006). The dominant allele is associated with the insertion of a retroelement in *VRN3* promoter (Yan et al. 2006). High expression level of *VRN3* promotes the transcription of *VRN1* and accelerates flowering (Li and Dubcovsky 2008; Yan et al. 2006). The molecular model for *VRN1/VRN2/VRN3* interactions suggests that *VRN3* is repressed by *VRN2* without vernalization treatment (Hemming et al. 2008; Yan et al. 2006). The low-temperature treatment caused the up-regulation of *VRN1* and the down-regulation of *VRN2* (Chen and Dubcovsky 2012); meanwhile, the low-level expression of *VRN2* also resulted in higher transcription level of *VRN3* (Distelfeld et al. 2009; Trevaskis et al. 2007). Therefore, low-temperature treatment advanced the flowering by directly up-regulating *VRN1* and down-regulating *VRN2* and indirectly up-regulating *VRN3*. *VRN-D4* gene originated by the insertion of an approximate 290-kb region from chromosome arm 5AL into the proximal region of chromosome arm 5DS. The inserted 5AL region included a copy of *VRN-A1* that carried distinctive mutations in its coding and regulatory regions. These shared mutations can be used to modulate vernalization requirements and to develop wheat varieties better adapted to different or changing environments (Kippes et al. 2015).

Vernalization requirement in common wheat is primarily controlled by three orthologous *VRN1* genes located in the middle of the long arms of chromosomes 5A, 5B, and 5D (Pugsley 1971; Pugsley 1972; Law et al. 1975; Stelmakh 1993; Galiba et al. 1995; Dubcovsky et al. 1998; Kato et al. 1999; Barrett et al. 2002; Iwaki et al. 2002; Tóth et al. 2003). Multiple alleles have been described within the *VRN-A1*, *VRN-B1*, and *VRN-D1*, based on their special structural characterizations and vernalization requirement as well as effect on flowering time. The different dominant alleles (*Vrn-A1a* and *Vrn-A1b*) of *VRN-A1* in common wheat were mostly caused by promoter mutations (Yan et al. 2004a; Fu et al. 2005). The most abundant one in common wheat, *Vrn-A1a*, has an insertion of a foldback repetitive element and a duplicated region in the promoter, resulting in the complete elimination of the vernalization requirement (Yan et al. 2004a). Dominant alleles (*Vrn-B1a*, *Vrn-B1b*, and *Vrn-B1c*) of *VRN-B1* commonly contain large deletions in the first intron which are responsible for a spring habit (Fu et al. 2005; Santra et al. 2009; Milec et al. 2012; Shcherban et al. 2012; Wang et al. 2014). Dominant allele *Vrn-B1c* possesses a higher level of transcripts and an earlier heading date compared with *Vrn-B1a* (Shcherban et al. 2013). Dominant alleles (*Vrn-D1a*, *Vrn-D1b*, and *Vrn-D1c*) of *VRN-D1* are main results of large deletion in the first intron and promoter mutation in common wheat (Yan et al. 2004a; Fu et al. 2005; Zhang et al. 2015). The dominant *Vrn-D1a* allele headed earlier than *Vrn-D1b* allele (Zhang et al. 2012). Zhang et al. (2015) indicated that a 174-bp insertion in *Vrn-D1c* promoter region contributed to the increasement in *VRN-D1* gene expression leading to early heading and flowering. Therefore, various alleles of *VRN1* genes contribute differences in vernalization requirements, growth habits, and heading time of wheat.

Cloning of wheat *VRN* genes has facilitated the development of a series of molecular markers for improving efficiency in identifying different vernalization response alleles (Yan et al. 2003, 2004a, 2006; Fu et al. 2005; Milec et al. 2012; Chen et al. 2013; Chu et al. 2008). The characteristic distributions of allelic combinations of *VRN* in some countries have been identified by the molecular markers (Fu et al. 2005; Zhang et al. 2008; Sun et al. 2009; Milec et al. 2013; Guo et al. 2015). Allelic variations at *VRN* genes in diverse eco-geography regions were different. Those results suggested that different combinations of *VRN* genes have an adaptive value to various environmental conditions (Iwaki et al. 2000, 2001). Therefore, further identification of new *VRN* alleles in wheat can enrich genetic diversity resources and guarantee the maximize grain production of wheat under different or changing environments.

Chinese wheats are mainly planted in 10 agro-ecological zones, which are further divided into 26 sub-zones based on wheat reaction to temperature, types

of growth habit, and wheat growing seasons (He et al. 2001; Zhuang 2003). This distribution condition shows that Chinese wheat cultivars are greatly diversified in vernalization response to various environments, and alleles of *VRN* genes are abundant in Chinese wheat cultivars (Zhuang, 2003). Recently, several new alleles of *VRN* genes have been found in Chinese wheat cultivars (Chen et al. 2013; Zhang et al. 2015). In the present study, a novel dominant allele *Vrn-B1d* from Chinese landrace cultivar Hongchunmai was cloned and characterized. Functional markers were developed to identify novel allele *Vrn-B1d*. The effect of this allele on heading time was revealed. Additionally, a multiplex PCR was developed to distinguish all five alleles at *VRN-B1* locus simultaneously. These results would provide useful germplasm resources and method for marker-assisted selection (MAS) in wheat breeding for adaptation of wheat to different and changing environments.

Materials and methods

Plant material

Hongchunmai, one of Chinese mini-core germplasms, is a spring landrace wheat cultivar originating from Manasi, Xinjiang, China. The combination of four *VRN* genotypes of Hongchunmai is *vrn-A1*, *Vrn-B1d*, *Vrn-D1b*, and *vrn-B3*. The 262 Chinese mini-core germplasms were obtained from Dr. Chenyang Hao at Chinese Academy of Agricultural Sciences. To reveal the effect of novel allele *Vrn-B1d* on heading date, 238 F_2 plants derived from a cross between Jing 411 (a Chinese winter cultivar with *vrn-A1*, *vrn-B1*, *vrn-D1*, and *vrn-B3*) and Hongchunmai were used for evaluation of heading time in the greenhouse under non-vernalizing (20–25 °C) and long day (16 h light) conditions. Chinese Spring nulli-tetrasomic lines N5AT5D, N5BT5D, and N5DT5B were used to validate the genome specificity of the designed primers. Cultivars Longmai 32 (*vrn-B1*), Ganmai 8 (*Vrn-B1a*), Longmai 34 (*Vrn-B1b*), and Baiyoumai (*Vrn-B1c*) were used as controls to confirm the accuracy and efficiency of the newly developed markers.

Sequencing and structural analysis of *VRN-B1* gene

Three primer pairs Ex1/B/F3 (gaagcggatcgagaacaaga) and Ex2/B/R3 (tagcgctcataccgttcaag), Ex2/B/F1 (tcttgaacggtatgagcgctactt) and Ex3/B/R1 (tgtctcaaccttcgccttcagtttcc), and Ex3/B/F2 (ggaaactgaaggcgaaggttgaga) and Ex8/B/R2 (tgcactgccgcatcctctgc) designed by Milec et al. (2012) were used to clone the complete *VRN-B1* sequence of Hongchunmai. The number next to "Ex" in the primers refers to the numerical order of exons. PCR was performed following Milec et al. (2012). The cloned PCR products were excised from agarose gel and purified using Quick DNA Extraction Kit. Purified products were ligated into the pGEM-T Easy vector and transformed into competent cells of the *Escherichia coli* DH-5α strain. More than three clones detected by each colony PCR for new allele were sequenced by Augct Company (http://www.augct.com). Sequence assembly of the new allele and multiple alignments with the known alleles *vrn-B1* (AY747604.1), *Vrn-B1a* (AY747603.1), *Vrn-B1b* (FJ766015), and *Vrn-B1c* (HQ130482) were performed by DNAMAN Version 8.0 software (https://www.lynnon.com/) to reveal the specific structural characterization of new allele.

Primer design and analysis of genotypes

Based on sequence alignments of the new allele and four known alleles at the *VRN-B1* locus, two forward primers (B/F-1 and B/F-2) and a common reverse primer (B/R) were designed by Primer 5 software to discriminate the new allele and other known alleles (Supplement 1). PCR reactions were conducted in the volume 20 μL containing 100 ng genomic DNA, 4 pmol of each primer (Supplement 1) and 10 μL 2 × ES Taq Master Mix (CWBIO, Beijing, China) (200 μM of each dNTP and 1.5 mM $MgCl_2$) using BIO-RAD T100 Thermal Cycler. The cycling conditions were as follows: 94 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1.5 min, and a final 8-min extension at 72 °C. PCR products were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized using UV light.

The multiplex PCR was developed with two forward primers (B/F-1 and B/F-2) and a common reverse primer (B/R). The PCR conditions were as described above.

VRN-B1-specific primers described by Fu et al. (2005) and Milec et al. (2012) were used to detect genotypes of 262 Chinese mini-core germplasms. *VRN-D1*-specific primers described by Fu et al. (2005) and Zhang et al. (2012) were used to screen the genotypes of the 238 F_2 plants.

Greenhouse experiment

In order to reveal the effect of novel allele *Vrn-B1d* on heading time, the F_2 plants from the Jing411/Hongchunmai cross and parents were grown in the greenhouse with the temperature between 20 and 25 °C (non-vernalizing condition) and a photoperiod condition for 16 h light (long day). The light resource in the day was natural daylight and incandescent lamps were used at night as supplementary light to extend photoperiod. Two hundred thirty-eight germinated F_2 seeds were sown in soil-filled pots with five plants each pot. Heading date was recorded at the time when the first ear of each plant half emerged from the flag leaf. Heading time was the number of days from sowing to heading date. The mean value and standard deviation analysis were used to reveal the average level and fluctuation of the heading time by Microsoft Excel software**.** The greenhouse data was analyzed by SPSS software to reveal the effect of new allele *Vrn-B1d* on the heading time of wheat.

Results

Molecular characterization of a novel allele *Vrn-B1d*

Two hundred sixty-two Chinese mini-core collections were tested with the PCR primers designed by Fu et al. (2005) and Milec et al. (2012) for *VRN-B1* gene (Supplement 1). Among these cultivars, 222 (84.7%), 29 (11.1%), 9 (3.4%), and 1 (0.4%) carried alleles *vrn-B1*, *Vrn-B1a*, *Vrn-B1b*, and *Vrn-B1c*, respectively. However, the spring landrace wheat cultivar Hongchunmai did not produce any PCR products using the primers. This indicated that Hongchunmai did not have an intact first intron sequence or the *Vrn-B1a*/*-B1b*/*-B1c* first intron deletion, suggesting the presence of a new allele at *VRN-B1* locus.

Three primer pairs designed by Milec et al. (2012) were used to clone the complete *VRN-B1* gene in Hongchunmai. Amplicon sizes of two primer pairs (Ex2/B/F1 and Ex3/B/R1, Ex3/B/F2 and Ex8/B/R2) from Hongchunmai were consistent with fragment sizes of control cultivars Longmai 32 (*vrn-B1*), Ganmai 8 (*Vrn-B1a*), Longmai 34 (*Vrn-B1b*), and Baiyoumai (*Vrn-B1c*) (Fig. 1). These results suggested that there was no different existence from the second to eighth exon region of Hongchunmai. However, amplicon sizes of primers Ex1/B/F3 and Ex2/B/R3 in Hongchunmai were different from those of control cultivars (Fig. 1). Further DNA sequencing indicated that the fragment amplified in Hongchunmai was 2828 bp. Multiple alignments (Supplement 2) showed that compared with recessive *vrn-B1*, there were differences existing in the first intron region of Hongchunmai including a large 6850-bp deletion (from 670 to 7519 bp, counting from the start of the intron 1 in the recessive *vrn-B1* allele), a small 187-bp deletion (from 7851 to 8037 bp), a unique SNP (T to C, 7845 bp), and a 4-bp mutation (TTTT to ACAA, from 7847 to 7850 bp). Meanwhile, the unique intron 1 region of Hongchunmai was also significantly different from the corresponding part of the other known dominant alleles (Fig. 2). It was clear to highlight the special composition of Hongchunmai compared with *Vrn-B1a*: a small 187-bp deletion (from 7851 to 8037 bp), a unique SNP (T to C, 7845 bp), and a 4-bp mutation (TTTT to ACAA, from 7847 to 7850 bp). There were still differences between *Vrn-B1d* and *Vrn-B1b*,*Vrn-B1c*. Compared with *Vrn-B1b*, novel allele contained a small 176-bp deletion (from 7862 to 8037 bp), a unique SNP (T to C, 7845 bp), and a 4-bp mutation (TTTT to ACAA, from 7847 to 7850 bp). Additionally, the structural differences between *Vrn-B1d* and *Vrn-B1c* were even more intricate: 544-bp deletion (from 127 to 670 bp), 432-bp replication fragment (from 8195–8626 to 7325–7756 bp), SNP (T to C, 8225 bp) of *Vrn-B1c* and a small 187-bp deletion (from 7851 to 8037 bp), a unique SNP (T to C, 7845 bp), and a 4-bp mutation (TTTT to ACAA, from 7847 to 7850 bp) of *Vrn-B1d*. Therefore, the new allele was designated as *Vrn-B1d* in Hongchunmai (submitted to NCBI no. MG242339). Because the new allele *Vrn-B1d* contained a deletion from 7851 to 8037 bp where the binding site of primer Intr1/B/R3 was located, no PCR products was generated using the primer pair Intr1/B/F and Intr1/B/R3 (Fu et al. 2005).

Molecular markers of *Vrn-B1d* allele

Firstly, two pairs of new primers (B/F-1 and B/R, B/F-2 and B/R) were designed by Primer 5.0 software (http://www.premierbiosoft.com/primerdesign/) for identification of the novel allele *Vrn-B1d* and other four known alleles at *VRN-B1* locus. Secondly, Chinese Spring nulli-tetrasomic lines N5AT5D, N5BT5D, and N5DT5B were used to validate the genome specificity of the designed primers. Generally,

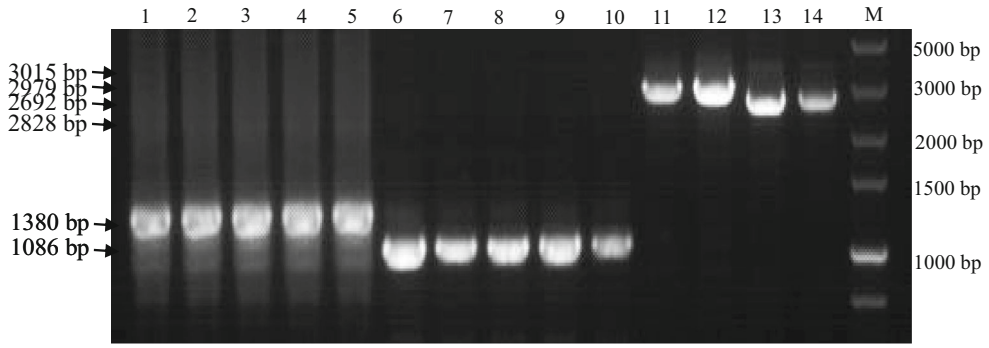

Fig. 1 The complete *Vrn-B1* sequences were amplified by the three primers in wheat cultivars. 1–5 PCR products amplified with Ex2/B/F1 and Ex3/B/R1, 6–10 Ex3/B/F2 and Ex8/B/R2, and 11–14 Ex1/B/F3 and Ex2/B/R3. M: DNA ladder DL 10000, 1, 6:Longmai32 (*vrn-B1*); 2, 7, 11:Ganmai 8 (*Vrn-B1a* (3015 bp)); 3, 8, 12:Longmai 34 (*Vrn-B1b* (2979 bp)); 4, 9, 13:Baiyoumai (*Vrn-B1c* (2692 bp)); 5,10, 14:Hongchunmai (*Vrn-B1d*(2828 bp))

we have to make sure whether the primers can specifically recognize the 5B chromosome on which the *VRN-B1* located. Afterwards, we should find out if the fragments were the same as the expected. Screening of Chinese Spring N5AT5D, N5BT5D, and N5 DT5B with the primers B/F-2 and B/R showed a consistent 870-bp fragment in Chinese Spring N5 AT5D and N5DT5B (Fig. 3b), while no PCR product in N5BT5D, which meaned primers could specifically recognize the 5B chromosome where the *VRN-B1* is located. Meanwhile, screening the control cultivars Ganmai 8, Longmai 34, Baiyoumai, and the landrace

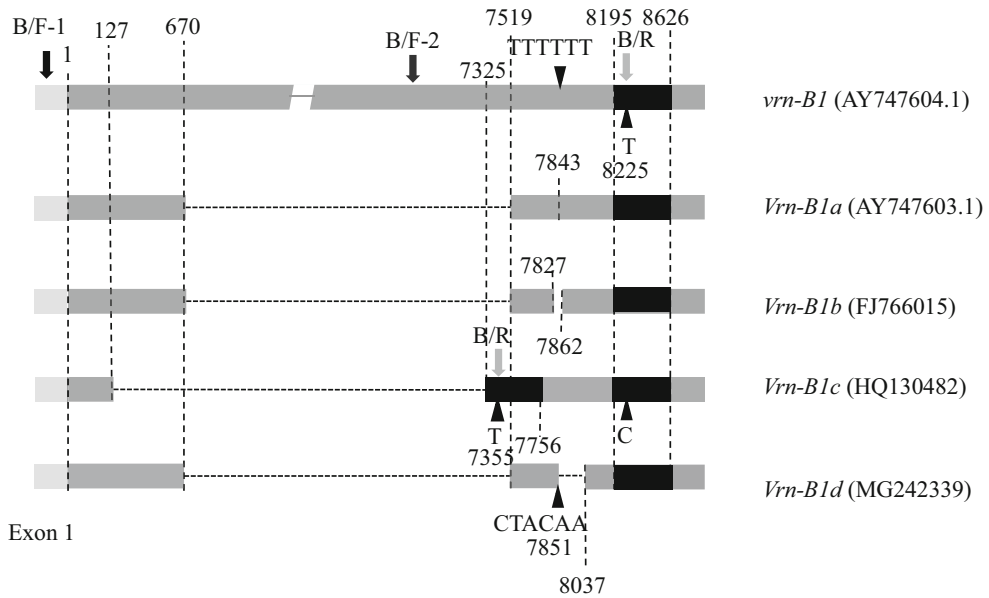

Fig. 2 Schematic comparison of the first intron variation of *Vrn-B1* alleles. Light and dark gray rectangles indicate exon 1 and intron 1, respectively. The dotted horizontal line indicates deletion. The dotted vertical line indicates the position of nucleotide from the start of intron 1. The filled triangles indicate the position of mutations. The *Vrn-B1d* allele in Hongchunmai contains a large 6850-bp deletion (from 670 to 7519 bp), a small 187-bp deletion (from 7851 to 8037 bp), a unique SNP (7845 bp) and a 4-bp mutation (from 7847 to 7850 bp) compared with the recessive allele *vrn-B1*. The black rectangle represents a region duplicated in allele *Vrn-B1c*. There is a 1-bp mutation (8225 bp, T to C) in allele *Vrn-B1c* compared with other four alleles, and a unique 432-bp duplicated region (from 7325 to 7756 bp). There is a 1-bp deletion (7843 bp) in allele *Vrn-B1a* compared with the recessive allele *vrn-B1*.The black arrow shows positions of forward primers B/F-1 and B/F-2, respectively. The gray arrow shows the position of reverse primer B/R. The primer B/R combines the 432-bp duplicated region (from 7325 to 7756 bp) in the allele *Vrn-B1c*

cultivar Hongchunmai with the primers B/F-1 and B/R correspondingly showed the expected fragments of 1541 bp, 1505 bp, 318 bp, and 1354 bp. These results meaned that the four cultivars contained *Vrn-B1a, Vrn-B1b, Vrn-B1c*, and *Vrn-B1d*, respectively (Fig. 3a). Therefore, the amplified fragments were the same as expected and the genotype data were consistent with the known genotypes for control cultivars, confirming the accuracy and efficiency of the designed molecular markers.

With the purpose to identify all the five alleles of *VRN-B1* gene in one PCR reaction, a multiplex PCR was developed based on the newly developed primers. One common reverse B/R and two forward primers B/F-1 and B/F-2 were applied to characterize all five alleles via successful amplification. The reaction components, amplification program, and electrophoresis condition of the multiplex PCR assays were shown in the "Materials and Method." Five alleles of *VRN-B1* gene could be discriminated by the multiplex PCR, according to the different sizes of fragments: 1541 bp (Ganmai 8; *Vrn-B1a*), 1505 bp (Longmai 34; *Vrn-B1b*), 318 bp (Baiyoumai; *Vrn-B1c*), 1354 bp (Hongchunmai; *Vrn-B1d*), and 870 bp (Jing 411; *vrn-B1*) (Fig. 4). These results were consistent with the single PCR amplifications, indicating that the multiplex PCR was accurate and reliable.

Effect of novel allele *Vrn-B1d* on heading time

To precisely reveal the effect of new allele *Vrn-B1d* on heading time and the association between vernalizaition genotypes and heading time, the F_2 population from the cross of Hongchunmai (*vrn-A1*, *Vrn-B1d*, *Vrn-D1b*, *vrn-B3*) and Jing 411 (*vrn-A1*, *vrn-B1*, *vrn-D1*, *vrn-B3*) were analyzed. Considering the different composition of the parental genotypes, there were nine possible vernalization genotypes in the F_2 population which were tested by PCR primers (Supplement 3). Meanwhile, the possible genotypes and the phenotypes of the F_2 population were shown (Supplement 3 and Table 1).

Among the F_2 plants carrying homozygous recessive allele (*vrn-D1vrn-D1*) at *VRN-D1* locus, plants with *Vrn-B1dVrn-B1d* genotype (69 d) headed significantly earlier than those with *Vrn-B1dvrn-B1* (79 d) ($p < 0.05$); plants with *vrn-B1vrn-B1* genotype did not head before 140 d (when the experiment was stopped) (Table 1). As for the other F_2 plants carrying dominant allele of *VRN-D1* in both heterozygous (*Vrn-D1bvrn-D1*) and homozygous (*Vrn-D1bVrn-D1b*) stages, a similar result that the heading time significantly advanced with the gradually increasing number of new dominant *Vrn-B1d* allele (from *vrn-B1vrn-B1*, *Vrn-B1dvrn-B1* to *Vrn-B1dVrn-B1d*) still existed, suggesting that new allele *Vrn-B1d* apparently advanced the heading time of wheat.

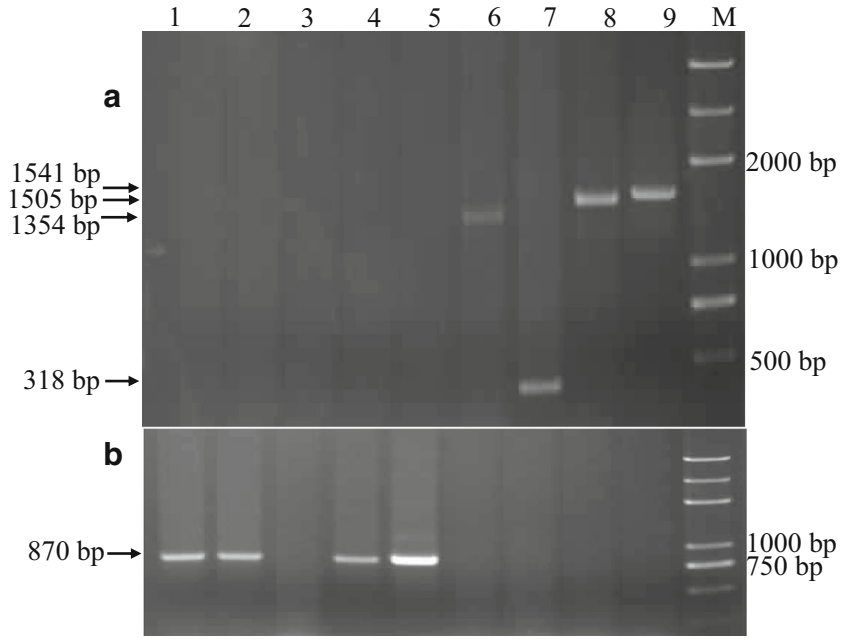

Fig. 3 Identification of *VRN-B1* genotypes by newly developed primers in some wheat cultivars. A: identification of dominant alleles of *VRN-B1* by the primers B/F-1 and B/R. B: identification of recessive allele of *VRN-B1* by the primers B/F-2 and B/R. M: DNA Ladder DL2000; 1: Chinese Spring (*vrn-B1*, 870 bp); 2: N5AT5D (*vrn-B1*, 870 bp); 3: N5BT5D (−, −); 4: N5DT5B (*vrn-B1*, 870 bp); 5: Longmai 32 (*vrn-B1*, 870 bp); 6: Hongchunmai (*Vrn-B1d*, 1354 bp); 7: Baiyoumai (*Vrn-B1c*, 318 bp); 8: Longmai 34 (*Vrn-B1b*, 1505 bp); 9:Ganmai 8 (*Vrn-B1a*, 1541 bp)

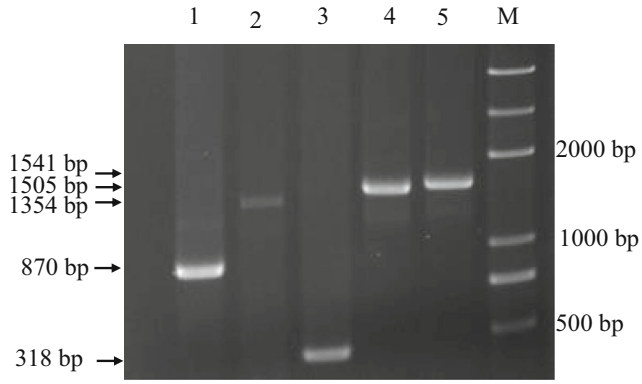

Fig. 4 Gel electrophoresis of multiplex PCR with two forward primers (B/F-1 and B/F-2) and one reverse primer B/R. M: DNA Ladder DL2000; 1: Jing 411 (*vrn-B1*, 870 bp); 2: Hongchunmai (*Vrn-B1d*, 1354 bp); 3: Baiyoumai (*Vrn-B1c*, 318 bp); 4: Longmai 34 (*Vrn-B1b*, 1505 bp); 5: Ganmai 8 (*Vrn-B1a*, 1541 bp)

Discussion

Molecular characterization, effect on heading time, and the contribution to the breeding of the novel allele *Vrn-B1d*

The previous studies revealed that promoter mutations and large deletions within the first intron of the *VRN-1* gene were associated with a spring growth habit (Yan et al. 2004a; Fu et al. 2005). Allelic variations of *VRN-B1* gene were only reported to occur in the first intron due to a large deletion in the dominant allele in spring wheat (Fu et al. 2005; Santra et al. 2009; Milec et al. 2012). In this study, we found a novel allele *Vrn-B1d* containing large and small deletion, a unique SNP, and a 4-bp mutation within the first intron in Chinese spring wheat landrace cultivar Hongchunmai (Fig. 2). As for the effect of the novel allele, *Vrn-B1d* had a stronger effect on the acceleration of heading under non-vernalizing condition than the recessive allele *vrn-B1* (Table 1). The first intron contains a region required to maintain repression of *VRN1* (von Zitzewitz et al. 2005; Fu et al. 2005; Cockram et al. 2007; Hemming et al. 2009). Fu et al. (2005) defined a 4.2-kb region of *VRN1* in the first intron as the vernalization "critical region." It was assumed that the "critical region" contained a binding site for a putative repressor that was down-regulated by vernalization (Fu et al. 2005; von Zitzewitz et al. 2005). The repressor could not bind the functional area, resulting in loss of function of the suppressing factor in the presence of large deletion within the first intron. The elimination of a repressor recognition site from the first intron might provide a simple explanation for early heading caused by the novel allele *Vrn-B1d*. The large deletion within the first intron was responsible for the spring wheat and the deletion fragments of each

Table 1 Effect of the combination of *VRN-B1* and *VRN-D1* genotypes in the F_2 population on heading time under non-vernalizing and long day conditions

Combination of genotype	Number of plants	Average heading time (d)
Vrn-D1bVrn-D1bVrn-B1dVrn-B1d	13	54 ± 4.4d
Vrn-D1bVrn-D1bVrn-B1dvrn-B1	24	63 ± 10.2c
Vrn-D1bVrn-D1bvrn-B1vrn-B1	11	65 ± 9.8c
Vrn-D1bvrn-D1Vrn-B1dVrn-B1d	36	62 ± 6.9c
Vrn-D1bvrn-D1Vrn-B1dvrn-B1	73	66 ± 12.4c
Vrn-D1bvrn-D1vrn-B1vrn-B1	24	71 ± 12.5b
vrn-D1vrn-D1Vrn-B1dVrn-B1d	15	69 ± 11.9bc
vrn-D1vrn-D1Vrn-B1dvrn-B1	27	79 ± 16.2a
vrn-D1vrn-D1vrn-B1vrn-B1	15	–

Different letters following the average heading time indicate significant differences (Tukey's test $p < 0.05$). – indicates that the data was not obtained before the experiment stopped

dominant allele were not the same. Therefore, there might be a potential difference in the heading time among the four dominant alleles. Additionally, the other phenotypes (plant height, ear length, leaf number, and so on) of these four dominant alleles might also be different. Another key point is that the novel first intron sequence of *Vrn-B1d* may be helpful for the further research of the first intron influence on the heading time. In the next stage, we would use qRT-PCR to determine the effect of this novel allele on wheat heading time in gene expression level.

Apart from the benefit of the scientific research, the novel allele still makes great contribution to the wheat breeding in Xinjiang. The temperature of Xinjiang is persistently low in the early stage of spring and drastically fluctuates in the late spring. Protecting wheat from the frost damage in the spring is an urgent problem in Xinjiang spring wheat production. Spring wheat cultivar Hongchunmai with the new allele *Vrn-B1d* was widely planted in this region, indicating that the *Vrn-B1d* genotype would be better adapted to Xinjiang. Therefore, the new allele *Vrn-B1d* may supply the beneficial germplasm resource for wheat breeding in similar climate regions as Xinjiang.

Development of gene-specific primers and effectiveness of the multiplex PCR

PCR-based assays could be used to directly identify genotypes by molecular markers without any interference from the environment during any period of wheat growth stages. Compared with the recessive allele *vrn-B1*, all the four dominant alleles contain large deletion in the first intron which would be applied to differentiate them from the recessive allele *vrn-B1* (Fig. 2, Supplement 2). For the four dominant alleles, the specific structural changes in the *Vrn-B1c* with 543-bp deletion (127–669 bp), 432-bp duplicated region (7325–7756 bp), and 1-bp mutation (8225 bp, T to C) can be used to separate this allele from the other three dominant alleles. Based on the different deletion positions in the *Vrn-B1b* (7827–7862 bp) and *Vrn-B1d* (7851–8037 bp) compared with *Vrn-B1a*, the three dominant alleles *Vrn-B1a*, *Vrn-B1b*, and *Vrn-B1d* can be distinguished. Therefore, reverse primer B/F-2 located in the 6850-bp deletion region was designed to specifically amplify the recessive allele *vrn-B1* with 870-bp in combination with reverse primer B/R located behind a 187-bp deletion region in *Vrn-B1d* (Fig. 2, Supplement 2). Since the *Vrn-B1c* allele contained a deletion from 127 to 669 bp, the first exon-based forward primer B/F-1 was designed to detect four dominant alleles of *VRN-B1* combined with common reverse primer B/R, which only combined the 432-bp duplicated region (from 7325 to 7756 bp) because of 1-bp mutation (8225 bp, T to C) in the allele *Vrn-B1c*.

With the combination of two forward primers (B/F-1 and B/F-2) and a common reverse primer (B/R), a multiplex PCR system was developed to discriminate all five alleles of *VRN-B1* simultaneously. By the use of the multiplex PCR system, five alleles of *VRN-B1* could be simply identified by one PCR-based reaction rather than two single PCR amplifications, which could reduce half of test time and sample requirements. The results tested by multiplex PCR system were consistent with those detected by single PCR, indicating that the multiplex PCR was accurate and reliable.

In this study, we identified a novel dominant allele *Vrn-B1d* in common wheat, developed two pairs of primers to identify *Vrn-B1d* and other four known alleles of *VRN-B1*, and established a multiplex PCR as an effective and reliable method for discriminating alleles at *VRN-B1* locus. In addition, we revealed that plants containing the *Vrn-B1d* allele showed obviously earlier heading date compared with those with recessive *vrn-B1* alleles, suggesting that *Vrn-B1d* was a dominant allele conferring the spring growth habit. This study provided a new wheat germplasm resource and useful markers for improvement of adaptability of wheat cultivars in breeding.

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References

Barrett B, Bayram M, Kidwell K, Weber WE (2002) Identifying AFLP and microsatellite markers for vernalization response gene *Vrn-B1* in hexaploid wheat using reciprocal mapping populations. Plant Breed 121:400–406

Chen A, Dubcovsky J (2012) Wheat TILLING mutants show that the vernalization gene *VRN1* down-regulates the flowering

repressor *VRN2* in leaves but is not essential for flowering. PLoS Genet 8:e1003134

Chen F, Gao M, Zhang J, Zuo A, Shang X, Cui D (2013) Molecular characterization of vernalization and response genes in bread wheat from the Yellow and Huai Valley of China. BMC Plant Biol 13:199

Chu CG, Xu SS, Friesen TL, Faris JD (2008) Whole genome mapping in a wheat doubled haploid population using SSRs and TRAPs and the identification of QTL for agronomic traits. Mol Breed 22:251–266

Cockram J, Chiapparino E, Taylor SA, Stamati K, Donini P, Laurie DA, O'sullivan DM (2007) Haplotype analysis of vernalization loci in European barley germplasm reveals novel *VRN-H1* alleles and a predominant winter *VRN-H1/VRN-H2* multi-locus haplotype. Theor Appl Genet 115: 993–1001

Distelfeld A, Li C, Dubcovsky J (2009) Regulation of flowering in temperate cereals. Curr Opin Plant Biol 12:178–184

Dubcovsky J, Lijavetzky D, Appendino L, Tranquilli G (1998) Comparative RFLP mapping of *Triticum monococcum* genes controlling vernalization requirement. Theor Appl Genet 97: 968–975

Fu DL, Szucs P, Yan LL, Helguera M, Skinner JS, von Zitzewitz J, Hayes PM, Dubcovsky J (2005) Large deletions within the first intron in *VRN-1* are associated with spring growth habit in barley and wheat. Mol Gen Genomics 273:54–65

Galiba G, Quarrie SA, Sutka J, Morgounov A, Snape JW (1995) RFLP mapping of the vernalization (*Vrn1*) and frost resistance (*Fr1*) genes on chromosome 5A of wheat. Theor Appl Genet 90:1174–1179

Guo X, Wang Y, Meng L, Liu H, Yang L, Zhou Y, Zhang H (2015) Distribution of the *Vrn-D1b* allele associated with facultative growth habit in Chinese wheat accessions. Euphytica 206:1–10

Hanocq E, Niarquin M, Heumez E, Rousset M, Gouis J (2004) Detection and mapping of QTL for earliness components in a bread wheat recombinant inbred lines population. Theor Appl Genet 110:106–115

He ZH, Rajaram S, Xin ZY, Huang GZ (2001) A history of wheat breeding in China. CIMMYT, Mexico

Hemming MN, Peacock WJ, Dennis ES, Trevaskis B (2008) Low-temperature and daylength cues are integrated to regulate FLOWERING LOCUS T in barley. Plant Physiol 147:355–366

Hemming MN, Fieg S, Peacock WJ, Dennis ES, Trevaskis B (2009) Regions associated with repression of the barley (*Hordeum vulgare*) *VERNALIZATION1* gene are not required for cold induction. Mol Gen Genomics 282:107–117

Iwaki K, Nakagawa K, Kuno H, Kato K (2000) Ecogeographical differentiation in east Asian wheat, revealed from the geographical variation of growth habit and *Vrn* genotype. Euphytica 111:137–143

Iwaki K, Haruna S, Niwa T, Kato K (2001) Adaptation and ecological differentiation in wheat with special reference to geographical variation of growth habit and *Vrn* genotype. Plant Breed 120:107–114

Iwaki K, Nishida J, Yanagisawa T, Yoshida H, Kato K (2002) Genetic analysis of *Vrn-B*1 for vernalization requirement by using linked *dCAPS* markers in bread wheat (*Triticum aestivum* L.). Theor Appl Genet 104:571–576

Kato K, Yamagata H (1988) Method for evaluation of chilling requirement and narrow-sense earliness of wheat cultivars. Jpn J Breed 38:172–186

Kato K, Miura H, Sawada S (1999) QTL mapping of genes controlling ear emergence time and plant height on chromosome 5A of wheat. Theor Appl Genet 98:472–477

Kippes N, Debernardi JM, Vasquez-Gross HA, Akpinar BA, Budak H, Kato K, Chao S, Akhunov E, Dubcovsky J (2015) Identification of the *VERNALIZATION 4* gene reveals the origin of spring growth habit in ancient wheats from South Asia. Proc Natl Acad Sci U S A 112:5401–5410

Law CN, Worland AJ, Giorgi B (1975) The genetic control of ear-emergence time by chromosomes 5A and 5D of wheat. Heredity 36:49–584

Li C, Dubcovsky J (2008) Wheat FT protein regulates *VRN1* transcription through interactions with *FDL2*. Plant J 55: 543–554

Loukoianov A, Yan L, Blechl A, Sanchez A, Dubcovsky J (2005) Regulation of *VRN-1* vernalization genes in normal and transgenic polyploid wheat. Plant Physiol 138:2364–2373

Milec Z, Tomkov L, Sumíková T, Pánková K (2012) A new multiplex PCR test for the determination of *Vrn-B1* alleles in bread wheat (*Triticum aestivum* L.). Mol Breed 30:317–323

Milec Z, Sumíková T, Tomková L, Pánková K (2013) Distribution of different *Vrn-B1* alleles in hexaploid spring wheat germplasm. Euphytica 192:371–378

Moon J, Suh SS, Lee H, Choi KR, Hong CB, Paek NC, Kim SG, Lee I (2003) The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant J 35:613–623

Pugsley AT (1971) A genetic analysis of spring-winter habit of growth in wheat. Aust J Agric Res 22:21–31

Pugsley AT (1972) Additional genes inhibiting winter habit in wheat. Euphytica 21:547–552

Santra DK, Santra M, Allan RE, Campbell KG, Kidwell KK (2009) Genetic and molecular characterization of vernalization genes *Vrn-A1*, *Vrn-B1*, and *Vrn-D1* in spring wheat germplasm from the Pacific Northwest region of the USA. Plant Breed 128:576–584

Shcherban AB, Efremova TT, Salina EA (2012) Identification of a new *Vrn-B1* allele using two near-isogenic wheat lines with difference in heading time. Mol Breed 29:675–685

Shcherban AB, Khlestkina EK, Efremova TT, Salina EA (2013) The effect of two differentially expressed wheat *VRN-B1* alleles on the heading time is associated with structural variation in the first intron. Genetica 141:133–141

Shitsukawa N, Ikari C, Shimada S (2007) The einkorn wheat (*Triticum monococcum*) mutant, maintained vegetative phase, is caused by a deletion in the *VRN1* gene. Genes Genet Syst 82:167–170

Stelmakh AF (1993) Genetic effects of *Vrn* genes on heading date and agronomic traits in bread wheat. Euphytica 65:53–60

Sun QM, Zhou RH, Gao LF, Zhao GY, Jia JZ (2009) The characterization and geographical distribution of the genes responsible for vernalization requirement in Chinese bread wheat. J Integr Plant Biol 51:423–432

Tóth B, Galiba G, Fehér E, Sutka J, Snape JW (2003) Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. Theor Appl Genet 107:509–514

Trevaskis B, Hemming MN, Dennis ES, Peacock WJ (2007) The molecular basis of vernalization-induced flowering in cereals. Trends Plant Sci 12:352–357

von Zitzewitz J, Szucs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casas A, Chen TH, Hayes PM, Skinner JS (2005) Molecular and structural characterization of barley vernalization genes. Plant Mol Biol 59:449–467

Wang X, Ju LP, Liu FJ, Zhang YY, Zhang F, Fu XJ, Feng Y, Zhang XK (2014) Vernalization effects of dominant alleles *Vrn-B1a* and *Vrn-B1b* and their distributions in cultivars from Yellow and Huai River Valleys facultative winter wheat zone (in Chinese). Acta Agron Sin 40(3):439–446

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene *VRN1*. Proc Natl Acad Sci U S A 100:6263–6268

Yan L, Helguera M, Kato K, Fukuyama S, Sherman J, Dubcovsky J (2004a) Allelic variation at the *VRN-1* promoter region in polyploid wheat. Theor Appl Genet 109:1677–1686

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004b) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1644

Yan L, Fu DL, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda S, Dubcovsky J (2006) The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. Proc Natl Acad Sci U S A 103:19581–19586

Yasuda S, Shimoyama H (1965) Analysis of internal factors influencing the heading time of wheat varieties. Ber Ohara Inst Landw Biol Okayama U 13:23–38

Zhang XK, Xia XC, Xiao YG, Dubcovsky J, He ZH (2008) Allelic variation at the vernalization genes *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Vrn-B3* in Chinese common wheat cultivars and their association with growth habit. Crop Sci 48:458–470

Zhang J, Wang Y, Wu S, Yang J, Liu H, Zhou Y (2012) A single nucleotide polymorphism at the *Vrn-D1* promoter region in common wheat is associated with vernalization response. Theor Appl Genet 125:1697–1704

Zhang X, Gao M, Wang S, Chen F, Cui D (2015) Allelic variation at the vernalization and photoperiod sensitivity loci in Chinese winter wheat cultivars (*Triticum aestivum* L.). Front Plant Sci 6:470

Zhuang QS (2003) Wheat improvement and pedigree analysis in Chinese wheat cultivars (in Chinese). China Agriculture Press, Beijing