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ORIGINAL PAPER

A single nucleotide polymorphism at the *Vrn-D1* promoter region in common wheat is associated with vernalization response

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Abstract Facultative wheat varieties adapt to a particular environment. But the molecular basis for the facultative growth habit is not clear relative to winter and spring growth habit. Two sets of wheat varieties were chosen for this study. Set 1 comprised ten spring accessions and Set 2 comprised ten facultative accessions. All accessions had been tested by the previously described allele-specific markers and shown having the same allelic composition of *vrn-A1 vrn-B1 Vrn-D1 and vrn-B3*. Here we examined whether differences in growth habit might be associated with as yet unidentified sequence variation at *Vrn-D1* locus. A region including the intron 1 deletion, the entire reading frame from a cDNA template and a part of promoter region of the dominant *Vrn-D1* gene in each of the accessions was sequenced, and a single nucleotide polymorphism was found between facultative accessions and spring accessions in the CArG-box at the promoter region. The novel allele in facultative accessions was designated as *Vrn-D1b*. The investigation of an F_2 population segregating for *Vrn-D1b* and *Vrn-D1*a (previously, *Vrn-D1*) in the greenhouse under long days without vernalization showed that the plants with *Vrn-D1b* homozygous allele headed 32 days later and had about three more leaves than the plants with *Vrn-D1a* homozygous allele. As *Vrn-D1b* has the same deletion in intron 1 as *Vrn-D1a*, and, in addition, a single nucleotide mutation at promoter region, and is associated with facultative growth habit, we suggest that the promoter mutation may modify the basal activity level of an allele of *VRN1* that is already active (due to the loss of segments in intron 1). Our finding further supports that both the promoter and intron 1 regulatory affect vernalization response and work independently.

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Introduction

Common wheat (*Triticum aestivum* L.) is one of the most widely distributed food crops in China, with growing area ranging from 22° to 48° in latitude, −150 to over 4,000 m in altitude, and under diverse cropping systems (Jin 1983). Wheat is sown in both autumn and spring. In autumn-sown wheat areas, the annual average temperatures range from 7 to 24 °C and the average temperatures in January range from −10.9 to 19 °C (Jin 1983). Ten wheat ecological zones have been classified based on different agroecological systems (Fig. 1). As the vernalization response is one of the important characters determining the basic adaptation of a genotype for a particular environment (Worland 1996; Snape et al. 2001), Chinese wheat varieties must be greatly diversified in vernalization response to adapt to the wide range of environments and therefore provide valuable genotypes for the research on the allelic variation in vernalization genes (Jin 1992; Zhang et al. 2008; Sun et al. 2009; Jiang et al. 2010).

The former studies showed that the vernalization response in wheat is genetically controlled by at least five loci *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, *Vrn4* and *Vrn-B3* (Pugsley 1971, 1972; McIntosh et al. 2008; Goncharov 2003; Yan et al. 2006; Yoshida et al. 2010). *Vrn-A1*, *Vrn-B1* and *Vrn-D1* are three major vernalization genes, which are located on the homologous chromosomes 5A, 5B and 5D in

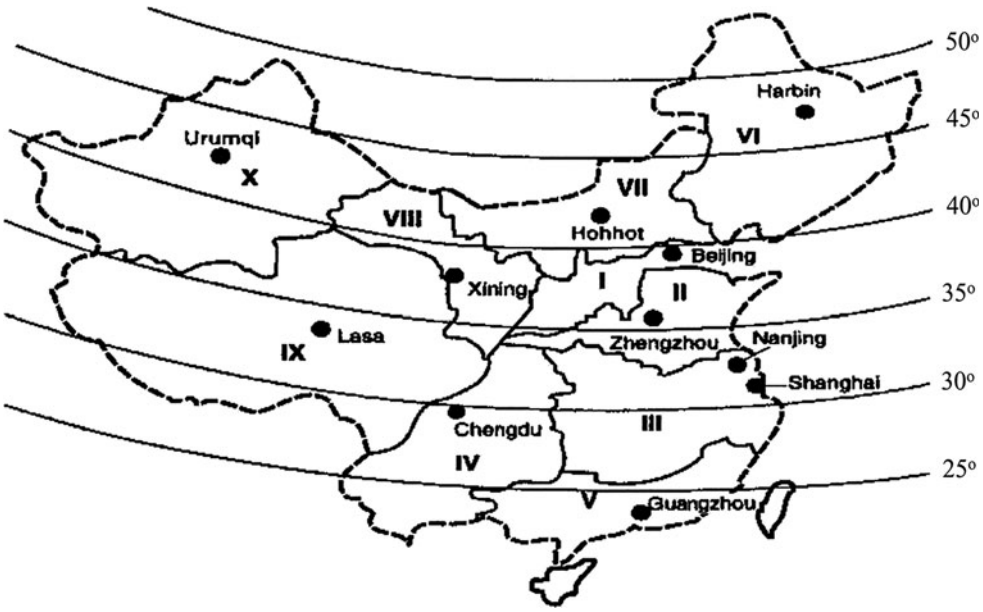

Fig. 1 The ten wheat ecological zones in China. I: Northern Winter Wheat Region; II: Yellow and Huai River Valley Winter Wheat Region; III: Middle and Low Yangtze River Valley Winter Wheat Region; IV: Southwestern Winter Wheat Region; V: Southern Winter Wheat Region; VI: Northeastern Spring Wheat Region; VII: Northern Spring Wheat Region; VIII: Northwestern Spring Wheat Region; IX: Qinghai-Tibetan Plateau Spring and Winter Wheat Region; X: Xinjiang Winter and Spring Wheat Region (Jin 1983)

common wheat, respectively (Pugsley 1971; Law et al. 1976; Iwaki et al. 2002; Yan et al. 2003). Three dominant alleles were found at *Vrn-A1* locus, i.e., *Vrn-A1a*, *Vrn-A1b* and *Vrn-A1c*, in which *Vrn-A1a* exerts the strongest effect on vernalization response, resulting in complete elimination of vernalization requirement (Pugsley 1971). *Vrn-A1a* has the foldback element insertion and duplication in the promoter region, whereas *Vrn-A1b* shows two mutations in the host direct duplication (HDD) region and 20 bp deletion in the 5′ UTR of the promoter region, and *Vrn-A1c* has a deletion in the first intron relative to the recessive *vrn-A1* allele (Yan et al. 2004; Fu et al. 2005). Both the dominant *Vrn-B1* and *Vrn-D1* genes have a large size deletion within their first intron, which is associated with spring growth habit, but no differences were found in promoter sequences compared to their respective recessive alleles (Yan et al. 2004; Fu et al. 2005).

The cloning of major wheat vernalization genes has facilitated the detection of these genes by the development of gene-specific markers or functional markers (Yan et al. 2003, 2004, 2006; Fu et al. 2005), which provides a unique opportunity to screen large collections of wheat cultivars for allelic diversity in vernalization genes. The previous studies on the allelic variation and its geographical distribution of vernalization genes in Chinese wheat varieties showed that the frequencies of different alleles varied greatly across wheat ecological zones, in which the dominant *Vrn-D1* allele was the most common allele among the dominant alleles in the nine out of ten ecological zones (Zhang et al. 2008; Sun et al. 2009; Jiang et al. 2010). Surprisingly, the varieties carrying the dominant *Vrn-D1* allele at *Vrn-D1* locus and the recessive alleles at other identified vernalization gene loci showed different vernalization requirement, ranging from typical spring growth habit with complete elimination of vernalization requirement to facultative growth habit with intermediate vernalization requirement relevant to winter varieties with strong vernalization requirement. Therefore, it is tempting to speculate that the differences in growth habit of these varieties with the dominant *Vrn-D1* allele could be related to the allelic variation at *Vrn-D1* locus.

Materials and methods

Plant materials

Two sets of wheat varieties from Zone II and Zone III were studied (Table 1). Set 1 comprised ten spring accessions and Set 2 comprised ten facultative accessions. All accessions had been tested by the previously described allele-specific markers for the allelic variation at *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Vrn-B3* loci (Yan et al. 2004, 2006; Fu et al. 2005) and shown having the same allele combination (Table 1). The information on growth habit of the accessions were from Zhuang (2003) and further confirmed by the author in summer sowing without vernalization, in which all accessions in Set 1 headed and all accessions in Set 2 failed to head in 110 days.

To establish F_2 population segregating for different alleles at *Vrn-D1* locus, Shimai 12 from Set 1 was crossed with Shi 4185 from Set 2. To compare the effects of allelic variation on vernalization requirement, 200 F_2 plants were grown in the greenhouse under a 18-h-day length regime and minimum temperature above 15 °C to avoid natural vernalization. The heading date and final number of leaves

Table 1 Allelic combination determined by the previously published allele-specific markers at the *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Vrn-B3* loci, growth habits and origins of the accessions (Zhuang 2003; Yan et al. 2004, 2006; Fu et al. 2005; Zhang et al. 2010)

Allele combination	Growth habit	Varieties and their origins
vrn-A1vrn-B1 Vrn-D1vrn-B3	Spring	Zone II: Shimai 12, Yumai 7, Yumai 18; Zone III: Yangmai 3, Yangmai 5, Yangmai 9, Yangmai 10, Yangmai 11, Yangmai 12, Yangmai 158
	Facultative	Zone II: Jimai 26, Shi 4185, Shi 91-5093, Ji 5265, Kenong 199, Shiluan 02-1, Shimai 14, Lumai 7, Bainong 3217, Yumai 13

on the main stem were recorded. 15 plants (5 plants from each genotype) were used for the gene expression analysis.

Cloning and sequencing

Genomic DNA was extracted from leaves using the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). A region including intron 1 deletion of the *Vrn-D1* gene was amplified using the primers Intr1/D/F: 5′-GTTGTCTGCCTCATCAAATCC-3′ (Fu et al. 2005) and Intr1/D/R3-1: 5′-CACGCAAGCAAGGAAATGGTG-3′. The reverse primer was designed to extend the amplified segment about 100 bp upstream of 3′ border compared with the original reverse primer 5′-GGTCACTGGTGGTCTGTGC-3′ by Fu et al. (2005). The annealing temperature for the PCR amplification was 56 °C. Besides, the open reading frame (ORF) fragments of D genome (AY747606) were amplified using the primers TaVRND1-1F (5′-ATGGGGCGCGGGAAGGTGCAG-3′) and TaVRND1-735R (5′-TCAGCCGTTGATGTGGCTCACCATC-3′). The annealing temperature was 65 °C. Furthermore, a part of promoter and 5′ UTR region of the *Vrn-D1* gene was amplified using the primers VRN1DF (5′-CGACCCGGGCGGCACGAGTG-3′) and VRN1R (5′-TGCACCTTCCCGCGCCCCAT-3′) (Yan et al. 2004). The annealing temperature for the PCR amplification was 65 °C. The PCR products were purified by TIANgel Midi Purification Kit (TIANGEN, Beijing, China), subcloned into the *pEASY*-T1 vectors (TransGen, Beijing, China) and then sequenced using M13 primers. Sequence analyses were performed in DNAMAN software (http://www.lynnon.com).

SNP marker design

The primers VRN1-SNP161CR (5′-AGGATGGCCAGGCCAAAACG-3′) and VRN1-SNP161AR (5′-AGGATGGCCAGGCCAAAACT-3′) were designed based on a single-nucleotide polymorphism (SNP) in the *Vrn-D1* promoter region. Primers VRN1DF and VRN1-SNP161CR were used to amplify a 612-bp promoter region of spring accessions, whereas primers VRN1DF and VRN1-SNP161AR to amplify the same size of promoter region of facultative accessions. The GC-rich promoter region was amplified using 2 × *EasyTaq* PCR SuperMix (TransGen, Beijing, China). The annealing temperatures for these two pairs of primers were both 65 °C.

Gene expression analysis

Total RNAs were extracted, using the TRIzol method (Invitrogen, Carlsbad, CA, USA) as described by Wilson et al. (2004), from the sprouting seedlings (4–5 cm coleptile length), germinated in the dark at about 20 °C, of all the accessions and F_2 population segregating for different alleles at *Vrn-D1* locus (5 plants of each genotype) mentioned above. Reverse transcriptions were performed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The open reading frame (ORF) fragments of A, B and D genome (AY747600, AY747604 and AY747606) were compared, and D genome-specific primers TaVRNDF (5′-CAAGATCAGACTCAGCCTCAAACA-3′) and TaVRNDR (5′-TAGCAACCGCAACATACACCAGA-3′) were designed by including inter-genome polymorphisms. *Vrn-D1* transcript levels were determined by quantitative reverse PCR (qRT-PCR) assay, using primers TaVRNDF and TaVRNDR. The expression of *Vrn-D1* gene was normalized to *ACTIN* using $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). *ACTIN* primers have been described previously (Trevaskis et al. 2006). qRT-PCR was performed in a LightCycler® 480II (Roche, Mannheim, Germany). All data presented are the average from three biological replications.

Results

Allelic variation at *Vrn-D1*

A fragment of 1,764 bp was amplified from the intron 1 of *Vrn-D1*, including 110 bp from the left border of the deletion and 1,654 bp from the right border of the deletion, and sequenced. Besides, the sequences of the entire open reading frame of *Vrn-D1* were obtained. In addition, a fragment of 772 bp upstream from the start codon in the promoter region to 20 bp in exon 1 of *Vrn-D1* gene was

amplified and sequenced. The sequence of the corresponding recessive *vrn-D1* allele was from GenBank (AY616457) and used as the wild type. By sequence comparison, all the accessions have the same deletion in the intron 1 and the identical D genome coding sequence as the recessive *vrn-D1*. There was no difference in the promoter region of *Vrn-D1* gene between the dominant *Vrn-D1* allele of spring accessions and the recessive *vrn-D1* allele. One SNP was found in the promoter region of *Vrn-D1* gene between the facultative accessions and the wild type, which was located 161 base pairs upstream from the translation initiation site ATG. Cytidylic acid at this site in the recessive *vrn-D1* allele was replaced by adenylic acid in the facultative accessions (Fig. 2). The SNP occurred in the CArG-box (Fig. 3), a recognition sequence for MADS-box proteins, previously reported as a highly conserved element among the wheat genomes (Yan et al. 2004). We designated the new allele as *Vrn-D1b* and the sequence of this allele was deposited in GenBank (JQ406528).

SNP marker analysis

Based on the single-nucleotide polymorphism in the *Vrn-D1* promoter region, we designed two primers using C or A as the 3′ terminal of the reverse primer to test all the spring and facultative accessions. Primers VRN1DF and VRN1-SNP161CR produced a 612-bp fragment of amplification production in the spring accessions, but not in the facultative accessions, whereas primers VRN1DF and VRN1-SNP161AR produced the same size fragment in the facultative accessions, but not in the spring accessions (Fig. 4). Therefore, the 612-bp fragment amplified by the two pairs of primers can be used as a molecular marker for selection of dominant *Vrn-D1a* (previously, *Vrn-D1*) for spring growth habit and *Vrn-D1b* for facultative growth habit, respectively.

In 200 F_2 plants from Shimai 12 × Shi 4185 cross grown in the greenhouse, 45 plants with the latest heading date and 45 plants with the earliest heading date were investigated for *Vrn-D1* allelic variation using the SNP marker described above and for number of leaves on the main stem (Table 2). The result showed that all the latest plants were *Vrn-D1b* homozygous, whereas all the earliest plants were *Vrn-D1a* homozygous. The plants with *Vrn-D1b* homozygous allele headed 32 days later and had 3.4 more leaves on average than the plants with *Vrn-D1a* homozygous allele.

Expression levels of *Vrn1-D1* gene

The qRT-PCR analyses were performed to compare the expression levels of *Vrn-D1* gene of the sprouting seedlings without cold treatment between Set 1 and 2 accessions and among F_2 population mentioned above (5 plants of each genotype). Higher level of expression of *Vrn-D1* gene was observed in the spring accessions compared with the facultative accessions (Fig. 5a). In F_2 plants, the expression of *Vrn-D1* gene in homozygous genotype (*Vrn-D1a*/*Vrn-D1a*) is the highest, followed by the heterozygous genotype (*Vrn-D1a*/*Vrn-D1b*), and the lowest in the genotype (*Vrn-D1b*/*Vrn-D1b*) (Fig. 5b).

Discussion

Fu et al. (2005) suggested that both the promoter and intron 1 regulatory sequences were required for the vernalization response and that mutations in either one of these two regulatory sites would be sufficient to eliminate or reduce the vernalization response, based on the findings that the dominant *Vrn-1* alleles for spring growing habit were frequently associated with either mutation in the promoter region (*Vrn-A1*) or deletion in the first intron (*Vrn-A1*, *Vrn-B1* and *Vrn-D1*) (Yan et al. 2004). In this study, a novel allele, designated as *Vrn-D1b*, having the same deletion in intron 1 as *Vrn-D1a*, and, in addition, a single-nucleotide mutation in CArG-box at promoter region was found to be associated with facultative growth habit, implying that the mutation in the promoter region may modify the basal activity level of an allele of *VRN1* that is already active (due to the loss of segments in intron 1). Our finding further supports that both the promoter and intron 1 regulatory affect vernalization response and work independently.

To investigate the effect of the SNP polymorphism in the promoter region on vernalization requirement, ideally, the promoter should be fused to reporter gene and activity examined in transgenic plants, with and without the mutation. The recent paper by Alonso-Peral et al. (2011) showed that the promoter of *VRN1* can drive high expression of Green Fluorescent Protein (GFP) in the leaves and shoot apex of barley. So this approach is valid.

VRN1 encodes MADS box transcription factors which bind specific site in the CArG-box in the promoter region of their target genes, resulting in the activation or repression of gene expression (Kane et al. 2007). We assume that the reduced effect of *Vrn-D1b* relative to *Vrn-D1a* is due to either the enhancement of the binding of a repressor, likely *VRN2* or *VRT2* or the disruption of the binding of an activator, likely *VRN3*. It seems more likely that a mutation in a conserved site would disrupt the binding of a transcription factor. If that were the case, the reduced effect of the allele is likely due to loss of activation. So the mutation presumably disrupts binding by MADS box floral promoter, not a repressor.

Trevaskis et al. (2003) reported that *WAP1*, the candidate for the *Vrn-1* gene, is strongly expressed in spring

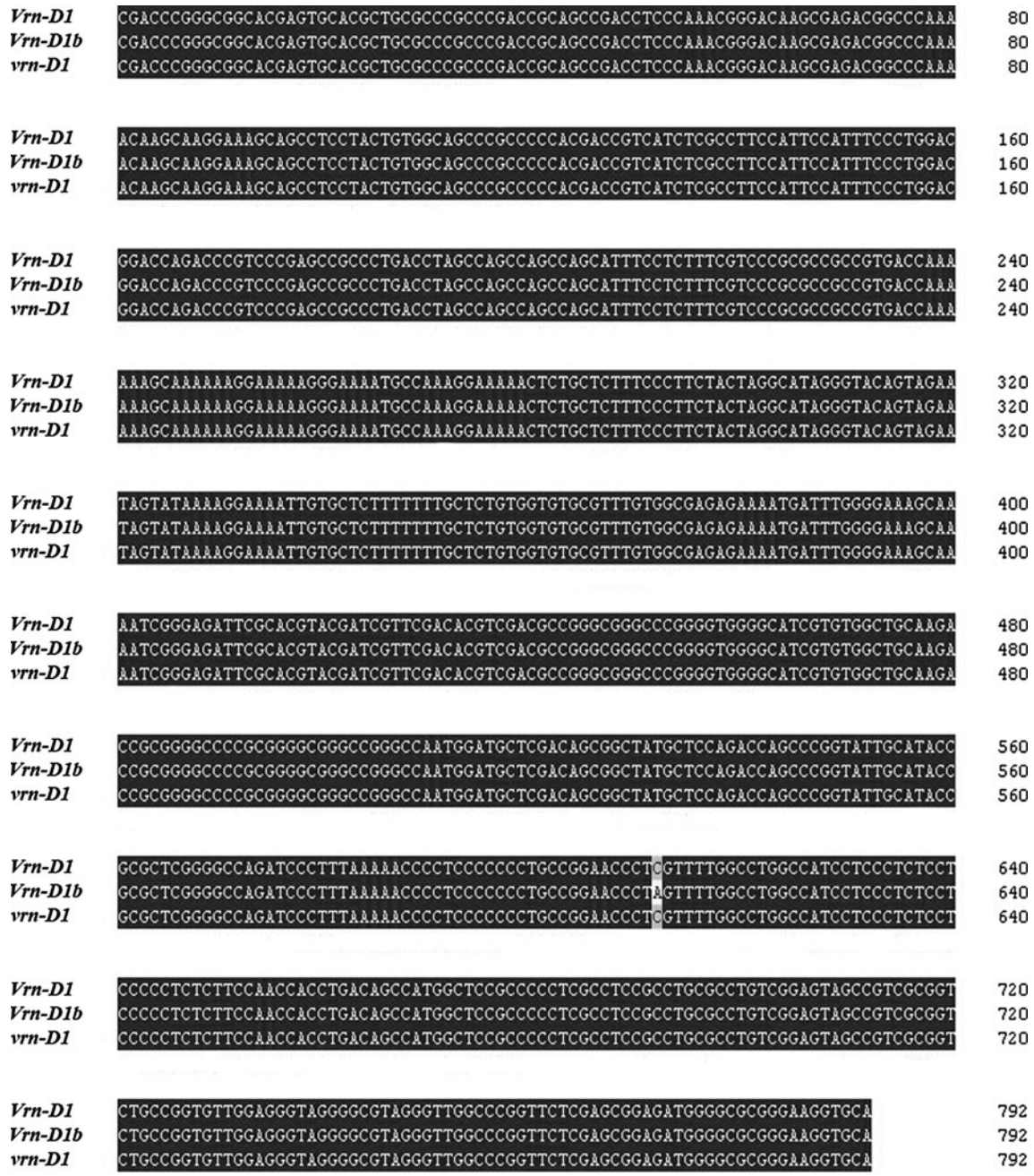

Fig. 2 Sequence alignment of a fragment of 772 bp upstream from the start codon in the promoter region to 20 bp in exon 1 of *Vrn-D1* gene amplified with genome-specific primers VRN1DF/VRN1R. The *Vrn-D1* and *Vrn-D1b* promoter fragments were obtained from spring and facultative accessions, respectively. The sequence of recessive *vrn-D1* allele was obtained from GenBank (AY616457) as the wild type. The C to A mutation, indicated in *light color*, was located 161 bp upstream from the initiation codon ATG

wheats and moderately expressed in semispring wheats, but is not expressed in winter wheat plants that have not been exposed to vernalization treatment and that vernalization strongly induces expression of *WAP1* and promotes flowering in winter wheats. Loukoianov et al. (2005) also reported that *VRN-1* was transcribed in leaves of the unvernalized spring isogenic lines but not in the unvernalized winter isogenic lines and that the dominant *Vrn-A1* allele was transcribed at earlier stage than the dominant *Vrn-B1* and *Vrn-D1* allele, which is in line with the different strength of spring growth habit. In this study, higher level of expression of *Vrn-D1* gene was observed in the spring accessions compared with the facultative accessions, and the transcription level is in line with the strength of spring growth habit in F_2 population segregating for *Vrn-D1a* and *Vrn-D1b*, indicating that different *VRN-1*

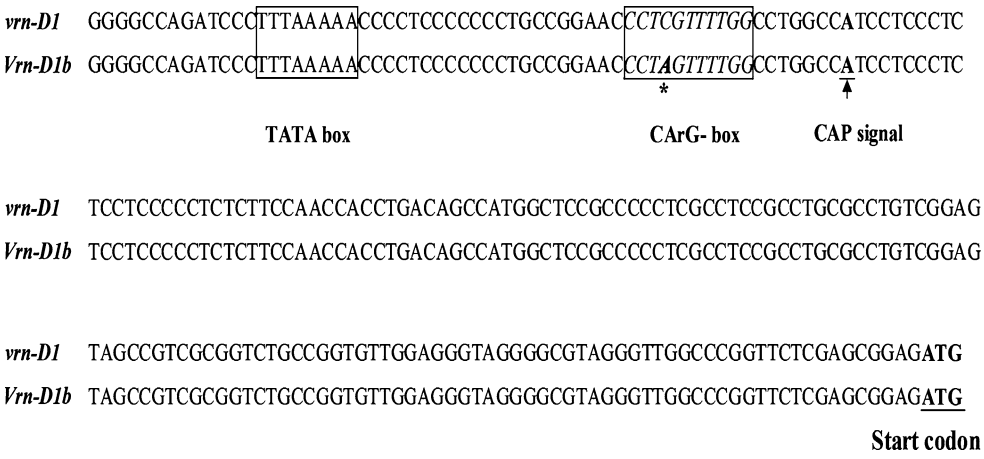

Fig. 3 Sequence difference in *Vrn-D1* promoter region between the recessive *vrn-D1* allele and the dominant *Vrn-D1b* allele. The ATG start codon is indicated in *bold* and *underlined*. The putative CAP signal for transcription initiation is indicated by an *arrow*. The TATA box is indicated in a *rectangle*. The CArG-box is indicated in *italic* and a *rectangle*, and the SNP in it is indicated by an *asterisk*

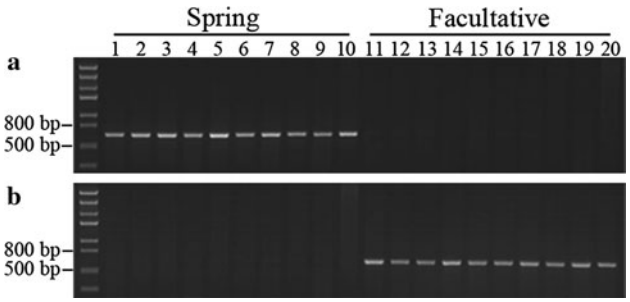

Fig. 4 **a** PCR amplification with primers VRN1DF/VRN1-SNP161CR showing the presence of a 612-bp fragment in the promoter region of *Vrn-D1* gene in spring varieties. **b** PCR amplification with primers VRN1DF/VRN1-SNP161AR showing the presence of 612 bp fragment in the promoter region of *Vrn-D1* gene in facultative varieties. 1, Shimai 12; 2, Yumai 7; 3, Yumai 18; 4, Yangmai 3; 5, Yangmai 5; 6, Yangmai 9; 7, Yangmai 10; 8, Yangmai 11; 9, Yangmai 12; 10, Yangmai 158; 11, Jimai 26; 12, Shi 4185; 13, Shi 91-5093; 14, Ji 5265; 15, Kenong 199; 16, Shiluan 02-1; 17, Shimai 14; 18, Lumai 7; 19, Bainong 3217; 20, Yumai 13

transcript levels can generate a continuum of flowering time in non-vernalized plants.

One of the assumptions for spring growth habit of the accessions with dominant *Vrn-D1a* gene in this study is that these spring accessions may carry more or different vernalization genes relative to the facultative accessions with dominant allele *Vrn-D1b*. Results of genetic analysis showed that the spring growth habit of Shimai 12, one of the spring accessions in Set 1, was controlled by a single dominant vernalization gene, *Vrn-D1a* (Zhang et al. 2010). Therefore, the difference in vernalization response between the accessions in Set 1 and 2 should be attributed to the allelic variation at *Vrn-D1* locus, which was further confirmed in the investigation of an F_2 population segregating for *Vrn-D1a* and *Vrn-D1b* allele in this study.

From 1982 to 1985 and from 1988 to 1990, Jin (1992) organized a nationwide research project on vernalization response and photoperiod response of Chinese wheat varieties across ten ecological zones in China. The results of this project demonstrated that there were consecutive changes in vernalization response from being highly sensitive, through various intermediate sensitive, to complete insensitive. The presence of *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, *Vrn-B3* and allelic variation at *Vrn-A1* locus in Chinese wheat varieties reported previously (Zhang et al. 2008; Sun et al. 2009; Jiang et al. 2010), together with the novel allele *Vrn-D1b* found in this study, provides an explanation from molecular basis for the phenomenon of great diversification in vernalization response. We speculate that more allelic variation at vernalization related loci, particularly at *Vrn-1*, may exist in Chinese wheat varieties.

Previous studies have shown that the dominant *Vrn-A1* allele was confined to Zone VI, VIII and X spring-sown

Table 2 Heading date and number of leaves on the main stem of F_2 plants from Shimai 12 × Shi 4185 cross

Allele	Heading date[a]			Number of leaves		
	Mean	Range	CV (%)	Mean	Range	CV (%)
Vrn-D1b	91.0	83 ~ 94	3.4	12.3	10 ~ 14	7.5
Vrn-D1a[b]	59.0	50 ~ 62	5.9	8.9	7 ~ 10	9.3
Difference	32**			3.4**		

** Significantly different at $P = 0.001$

[a] Days from seedling emergence to heading

[b] *Vrn-D1a* previously designated as *Vrn-D1*

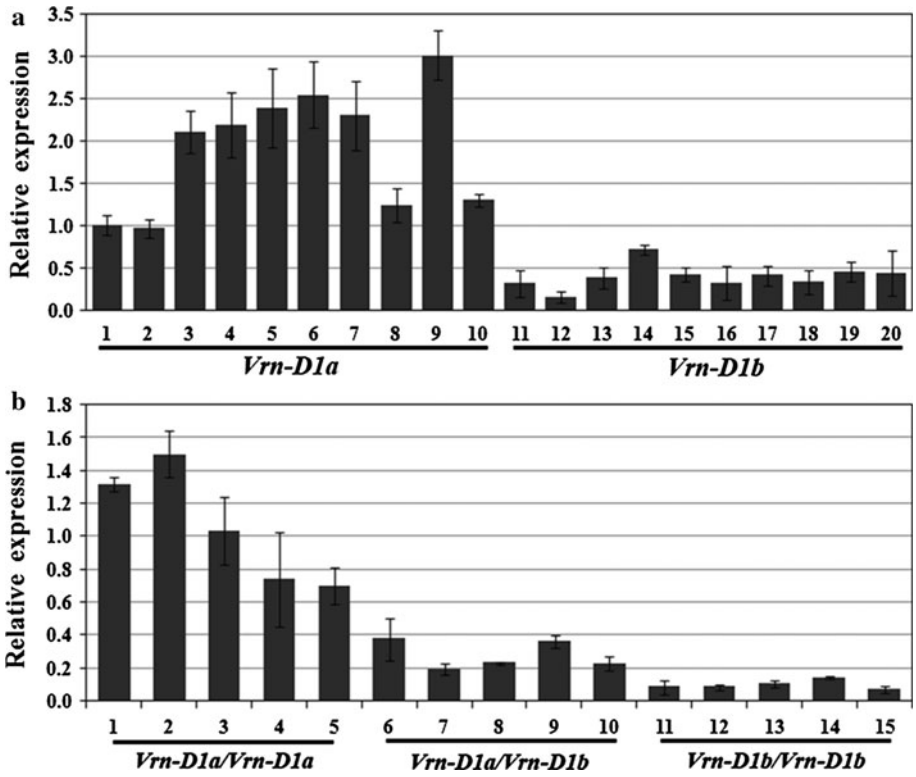

Fig. 5 Relative expression level of *Vrn-D1* gene of the sprouting seedlings without cold treatment. **a** Relative expression level of *Vrn-D1* gene of the sprouting seedlings without cold treatment in spring and facultative accessions. 1, Yangmai 158; 2, Yumai 18; 3, Yangmai 3; 4, Yumai 7; 5, Shimai 12; 6, Yangmai 5; 7, Yangmai 9; 8, Yangmai 10; 9, Yangmai 11; 10, Yangmai 12; 11, Bainong 3217; 12, Yumai 13; 13, Shi 91-5093; 14, Shi 4185; 15, Lumai 7; 16, Ji 5265; 17, Jimai 26; 18, Kenong 199; 19, Shiluan 02-1; 20, Shimai 14. **b** Relative expression level of *Vrn-D1* gene of the sprouting seedlings without cold treatment in F_2 population segregating for *Vrn-D1a* and *Vrn-D1b* (5 plants of each genotype). Expression is shown relative to *ACTIN*. *Error bars* show standard error for three biological replicates

regions, whereas the dominant *Vrn-D1* allele was widely distributed throughout China (Zhang et al. 2008; Sun et al. 2009; Jiang et al. 2010). This study indicated that the allelic variation at *Vrn-D1* locus, in addition to different vernalization gene loci, may provide additional value to the adaptation of a genotype for a particular environment. For example, Set 1 varieties with *Vrn-D1a* were mainly grown in Zone III, which belongs to subtropical area, whereas Set 2 varieties with *Vrn-D1b* were mainly grown in Zone II, belonging to temperate area. In Zone II, where the double cropping system is widely adopted, wheat, often following corn or soybean, is sown in early to middle October, which is optimal for the sowing of facultative wheat, but too early for the sowing of spring wheat. If spring varieties were to be sown early in Zone II, a period of prolonged warm temperatures could lead to premature development of the delicate floral primordia which would then be susceptible to winter frost damage and lead to severe yield loss. Compared with winter wheat, facultative wheat usually has earlier maturity which is essential to permit a sufficient grain filling period before the onset of particularly hot and dry summer conditions and timely sowing of the following crops (Jin 1992). Some spring varieties such as Yumai 7 and Yumai 18 in Set 1, carrying dominant *Vrn-D1a* allele, which were earlier in maturity than facultative varieties, were grown in southern part of Zone II too, when the sowing is delayed, which is often the case if cotton or other late harvested crops are grown as the previous crops.

Accurate and reliable selection for a certain growth habit is very important in breeding program. Traditionally, breeders select for the growth habit according to observation of seedling performance in the field, which sometimes led to incorrect classification. For example, Shimai 12 in Set 1, a spring variety with the seedling characteristics of facultative wheat was misclassified as facultative wheat when it was released in the northern part of Zone II, where only facultative and winter wheat are grown, which led to severe yield loss as the result of cold damage. The molecular marker developed in this study for the quick detection for the facultative growth habit, together with the molecular markers reported previously (Yan et al. 2004, 2006; Fu et al. 2005), will efficiently facilitate the selection on the growth habit for a particular environment.

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