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ORIGINAL PAPER

Vrn-D4 is a vernalization gene located on the centromeric region of chromosome 5D in hexaploid wheat

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Abstract Natural variation in wheat requirement of long exposures to cold temperatures to accelerate flowering (vernalization) is mainly controlled by the *Vrn-1*, *Vrn-2*, *Vrn-3*, and *Vrn-4* loci. The first three loci have been well characterized, but limited information is available for *Vrn-4*. So far, natural variation for *Vrn-4* has been detected only in the D genome (*Vrn-D4*), and genetic stocks for this gene are available in Triple Dirk (TDF, hereafter). We detected heterogeneity in the *Vrn-1* alleles present in different TDF stocks, which may explain inconsistencies among previous studies. A correct TDF seed stock from Japan carrying recessive *vrn-A1*, *vrn-B1*, and *vrn-D1* alleles was crossed with three different winter cultivars to generate F_2 mapping populations. Most of the variation in flowering time in these three populations was controlled by a single locus, *Vrn-D4*, which was mapped within a 1.8 cM interval flanked by markers *Xcfd78* and *Xbarc205* in the centromeric region of chromosome 5D. A factorial ANOVA for heading time using *Vrn-D4* alleles and vernalization as factors showed a significant interaction ($P < 0.0001$), which confirmed that the *Vrn-D4* effect on flowering time is modulated by vernalization. Comparison of the different Triple Dirk stocks revealed that *Vrn-B1*, *Vrn-D1*, and *Vrn-D4* all have a small residual response to vernalization, but *Vrn-D4* differs from the other two in its response to short vernalization periods. The precise mapping and characterization of *Vrn-D4* presented here represent a first step toward the positional cloning of this gene.

T. Yoshida and H. Nishida contributed equally to the work.

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Introduction

Flowering at an optimal time is very important for plant reproductive success. To achieve this, plants monitor seasonal changes using environmental cues, such as differences in day length (photoperiod) and the exposure to low temperatures for extended periods of time (vernalization). These seasonal cues are integrated with additional information from the environment (e.g., water or nutrient stresses, limited root space, etc.) and from internal cues (e.g., age of the plant) to determine the initiation of the reproductive phase. The regulation of this transition is particularly critical for annual plants, such as the temperate cereals, since the transition to the reproductive phase is intimately associated with senescence and plant death.

The requirement for vernalization is particularly important for winter cereals to avoid cold injury of the sensitive floral organs during the winter. In wheat, vernalization requirement is controlled by four major genes designated *Vrn-1*, *Vrn-2*, *Vrn-3,* and *Vrn-4* (reviewed in Distelfeld et al. 2009a; Flood and Halloran 1986; Trevaskis et al. 2007; Worland et al. 1987). The first three genes have been identified using map-based cloning approaches and

validated using mutants and transgenic plants (Yan et al. 2003, 2004b, 2006).

The *Vrn-1* gene encodes a MADS-box transcription factor closely related to the Arabidopsis *AP1/FRUITFULL* family (Yan et al. 2003), which is essential for the transition from the vegetative to reproductive stage in wheat (Shitsukawa et al. 2007). Natural insertions or deletions (indels) in regulatory regions of the three homoeologous genes found in hexaploid wheat (*Vrn-A1, Vrn-B1*, and *Vrn-D1*) are associated with dominant alleles for spring growth habit (Fu et al. 2005; Yan et al. 2004a). During vernalization, these regulatory regions show changes in histone methylation and acetylation associated with the transition between repressed and active chromatin states (Oliver et al. 2009). Different combinations of *Vrn-A1, Vrn-B1*, and *Vrn-D1* dominant alleles are the most common sources of spring growth habit among landraces and commercial cultivars of polyploid wheat around the world (Fu et al. 2005; Iqbal et al. 2007; Iwaki et al. 2000, 2001; Stelmakh 1987b; Yan et al. 2004a; Zhang et al. 2008).

The *Vrn-2* locus includes two linked and related proteins designated ZCCT1 and ZCCT2, characterized by the presence of a putative zinc finger and a CCT domain (Yan et al. 2004b). Deletions and mutations involving both ZCCT1 and ZCCT2 genes are frequent in diploid wheat and barley and are associated with recessive alleles for spring growth habit (Dubcovsky et al. 2005; Hemming et al. 2009; Yan et al. 2004a). Among the tetraploid wheat species, the *Vrn-B2* gene is generally functional whereas the *Vrn-A2* gene is not (Distelfeld et al. 2009b). Since *Vrn-2* is the only locus with a dominant winter growth habit, at least one functional copy of *Vrn-2* combined with homozygous recessive alleles at all three *Vrn-1* loci is required to confer winter growth habit in hexaploid wheat.

The *Vrn-B3* locus (formerly known as *Vrn-5* or *Vrn-B4*; McIntosh et al. 2003) is homologous to the Arabidopsis *FT* gene (Yan et al. 2006). This dominant allele, found in the variety Hope, is associated with the insertion of a transposable element in the *Vrn-B3* promoter. Natural variation at the *Vrn-A3* and *Vrn-D3* loci has also been described in hexaploid wheat (Bonnin et al. 2008). *Vrn-3* promotes the transcription of *Vrn-1* and accelerates flowering (Li and Dubcovsky 2008; Yan et al. 2006). In several species, it has been shown that FT can travel from the leaves to the shoot apex through the phloem (Corbesier et al. 2007; Lin et al. 2007; Tamaki et al. 2007). In wheat, the VRN3 protein interacts with FDL2, which binds to the *Vrn-1* promoter (Li and Dubcovsky 2008).

Current models of flowering regulation in the temperate cereals suggest that, before vernalization, *Vrn-3* is repressed by *Vrn-2* (Hemming et al. 2008; Yan et al. 2006). Long exposures to cold temperature result in the up-regulation of *Vrn-1* and the down-regulation of *Vrn-2* in the leaves. The release from the *Vrn-2* repression results in higher transcript levels of *Vrn-3* and the promotion of *Vrn-1* above the threshold levels required for flower induction (Distelfeld et al. 2009a; Trevaskis et al. 2007).

In contrast to the previous three vernalization genes, little is known about *Vrn-4*. The allele for early flowering was originally identified in the Australian cultivar Gabo (Knott 1959; Pugsley 1972), and was backcrossed into Triple Dirk to develop an isogenic line designated TDF (Pugsley 1972). This locus was assigned to chromosome 5D by monosomic analysis (Kato et al. 1993) and is currently designated as *Vrn-D4* (formerly known as *Vrn4* or *Vrn-D5*; McIntosh et al. 2003). This locus was later mapped closely linked to SSR marker *Xgdm3* on the centromeric region of chromosome 5D (Kato et al. 2003). Natural variation for flowering time at the centromeric region of homoeologous group 5 chromosomes has been found, so far, only in the D genome. While some studies have questioned the existence of *Vrn-D4* (Maystrenko 1980; Stelmakh 1987b) or its chromosome location (Goncharov 2003), abundant evidence is presented here supporting its 5D chromosome location.

Using genetic analyses, Iwaki et al. (2000, 2001) found the *Vrn-D4* allele for spring growth habit in many spring wheat landraces from different parts of the world (55 out of 272), with a higher frequency in India and neighboring regions. Therefore, the *Vrn-D4* locus appears to be an important contributor to variation in flowering time in the hexaploid wheat germplasm and the identification of the gene responsible for these differences may have practical applications in breeding. In addition, the identification of *Vrn-4* is important to advance our understanding of the vernalization pathway in the temperate cereals, which appear to have evolved independently of the vernalization pathway in the dicot species (Yan et al. 2004b). The mapping results from this study represent an initial step toward the identification of this gene.

Materials and methods

Plant materials

Two different stocks of the near isogenic line Triple Dirk F (TDF) were used in this study (Table 1). The first one was obtained from Dr. T. Gotoh and was maintained at Okayama University, Japan (TDF-J, hereafter), and the second one was obtained from K. Campbell at Washington State University, USA (TDF-US, hereafter). TDF-J is the same line used by Kato et al. (2003) for the preliminary map of *Vrn-D4*. The *Vrn-1* alleles present in each stock were determined using available molecular markers (Fu et al. 2005; Yan et al. 2004a).

Table 1 *Vrn* genotype of Triple Dirk (TD) NILs and winter varieties/line used in this study

Line	Genotype[a]				Growth habit[b]	
TDF-J	*Vrn-D4*	*vrn-A1*	*vrn-B1*	*vrn-D1*	S	Triple Dirk NIL, Japan
TDF-US	*vrn-D4*	*Vrn-A1*	*Vrn-B1*	*vrn-D1*	S	Triple Dirk NIL, USA
TDD	*vrn-D4*	*Vrn-A1*	*vrn-B1*	*vrn-D1*	S	Triple Dirk NIL
TDB	*vrn-D4*	*vrn-A1*	*Vrn-B1*	*vrn-D1*	S	Triple Dirk NIL
TDE	*vrn-D4*	*vrn-A1*	*vrn-B1*	*Vrn-D1*	S	Triple Dirk NIL
TDC	*vrn-D4*	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	Triple Dirk NIL
Akakawaaka	*vrn-D4*	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	Japanese winter variety
Hayakomugi	*vrn-D4*	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	Japanese winter variety
CS($5D_{5402}$)	*vrn-D4*	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	Chinese Spring substitution line with *Ae. tauschii* 5D chromosome

[a] *vrn* recessive allele for winter growth habit, *Vrn* dominant allele for spring growth habit

[b] S and W indicate spring growth habit and winter growth habit, respectively

Three populations were developed for the mapping of *Vrn-4*. The initial mapping populations included 144 F_2 plants from the cross between TDF-J and Akakawaaka, a Japanese winter cultivar (Table 1). The limited level of polymorphism observed between the parental lines of this cross prompted the development of two additional populations. The second population included 258 F_2 plants from the cross between the Japanese winter cultivar Hayakomugi (Table 1) and TDF-J. The third population (159 F_2 plants) was developed from the cross between TDF-J and a substitution line of chromosome 5D from synthetic wheat 5402 in Chinese Spring, henceforth CS($5D_{5402}$) (Table 1). Synthetic RL5402 was generated by Dr. E. R. Kerber (Canada Agriculture Research Station, Winnipeg, Manitoba, Canada) from the cross between Tetra Cantach and *Ae. tauschii* (Kerber 1964). The CS($5D_{5402}$) line was developed by Dr. Jan Dvorak (University of California, Davis, USA), who kindly provided us the seeds. Synthetic 5402 was selected among the nine different synthetic lines characterized in the NSF-Wheat-SNP project (http://wheat.pw.usda.gov/SNP/new/index.shtml) because of its high level of polymorphisms with non-synthetic wheats.

The first two populations were analyzed in Japan, and flowering time was determined as the number of days from sowing to flag leaf unfolding. The third population and the interaction studies were performed in the US and flowering time was determined as number of days from sowing to heading. Progeny tests were conducted using F_3 seeds to validate the genotyping of F_2 plants with critical recombination events flanking the *Vrn-D4* locus or with intermediate flowering times in the F_2 generation.

Nulli-tetrasomic lines for chromosome 5D, ditelosomic line Dt5DL, and deletion lines for chromosome 5D with break point 5DS2, 5DS5, 5DS1, 5DL1, 5DL9, and 5DL5 were used to determine the arm location and physical position of the markers in the chromosome (Endo and Gill 1996; Linkiewicz et al. 2004; Sears and Steintz-Sears 1978). The TDF-J stock was compared with other Triple Dirk spring near isogenic lines (NILs) carrying the *Vrn-A1* (TDD), *Vrn-B1* (TDB), *Vrn-D1* (TDE) and the winter NIL with recessive alleles for all the previous genes (TDC) (Table 1).

To study the interaction between *Vrn-D4* alleles and vernalization, two F_2 plants homozygous for the *Vrn-D4* allele (TDF) and two homozygous for the *vrn-D4* allele (Hayakomugi) were selected from the TDF × Hayakomugi segregating population. Ten F_3 seeds from each plant were sown in individual pots (20 plants for each allele, total 40 plants). Half of the plants for each allele were vernalized for 6 weeks at 4°C and the other half were kept in a greenhouse at 20–25°C under the same photoperiod (16 h light). Heading times were recorded at the time of spike emergence.

Growth conditions

The F_2 population from a cross between TDF-J and Akakawaaka was grown at constant temperature 20°C (non-vernalizing condition) and continuous light (24 h) in a growth chamber (LH-350SP, Nippon Medical & Chemical Instruments Co. Ltd., Japan). Light source was fluorescent lamps and photon flux density was ca. 160 μmol/m^2/s. Planting density was one plant per 2.8 × 4.3 cm^2 in a plastic tray (48 × 33 × 7 cm) filled with the 1:1 mixture of soil and bark compost.

The F_2 population from a cross between TDF-J and Hayakomugi and their progeny F_3 lines were grown in the same growth chamber using the same conditions as above except for the adjustment of the photoperiod to 16 h of light and 8 h of dark (long day), and planting density 2.8 × 5.9 cm^2.

The F_2 population from a cross between TDF-J and CS($5D_{5402}$) was grown in the greenhouse where air temperature was kept over 20–25°C (non-vernalizing condition) and photoperiod was 16 h. Light source in the day was natural daylight and at night incandescent lamps were used as supplementary light to extend photoperiod. Individual seeds were sown in soil-filled half-gallon pots.

To compare the vernalization response of TDF relative to other Triple Dirk NILs (Table 1), seeds were soaked in

water at 4°C for 24 h and subsequently kept at 20°C for 24 h for germination. Six germinated seeds were planted for each of the eight treatments, which varied from 0 to 35 days at 2°C (5-day intervals, long days). After the vernalization treatments, plastic trays were transferred to the growth chambers under the same conditions as described above for the TDF-J × Akakawaaka mapping population until flag leaf unfolding. Plants for the non-vernalization control (0 days) were transferred to the growth chamber immediately after germination. Days from sowing to flag leaf unfolding were calculated as described before (Kato and Yamagata 1988). This method corrects for the slower growth at lower temperatures, so flowering time becomes approximately constant among fully vernalized plants irrespective of the duration of vernalization treatment.

Molecular markers and data analyses

Genomic DNA was extracted from young leaves of individual plants using the CTAB method (Murray and Thompson 1980). The *Vrn-1* genotype of different TDF stocks was determined by PCR using primers described before (Fu et al. 2005; Yan et al. 2004a). Marker *XBG313707* was developed from EST BG313707. D genome-specific primers BG313707_cpF1 (5′-GCTTCCAGACATCGGTCATT-3′) and BG313707D_R1 (5′-CACCACCAGTAACCCAG CC-3′) were used to sequence the critical recombinant lines and map a single nucleotide polymorphism (SNP).

Seven microsatellite markers, *Xcfd81*, *Xcfd78*, *Xcfd67*, *Xgdm68, Xbarc205*, *Xwmc318*, and *Xgdm3*, were used for genetic mapping (http://wheat.pw.usda.gov). PCR amplifications were performed in a 10 µl volume containing 1 µl of PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 1.5 mM of $MgCl_2$, 0.25 units of *Taq* polymerase (Sigma, USA), 0.2 mM of dNTP, 0.5 µM of primer, and 50 ng of template DNA.

PCR products for the *Vrn-1* alleles were separated in 1.2% agarose gels, and those from the SSR markers were separated in 6–18% polyacrylamide gels. PCR products were visualized with ethidium bromide. PCR conditions for the different microsatellite markers included a 95°C denaturing step for 3 min, followed by 35 cycles of 95°C for 30 s, 58–60°C annealing (depending on microsatellite marker) for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. Annealing temperatures for the different markers were as follows: 57°C for *Xcfd78, Xcfd81*, and *Xgdm68*; 60°C for *Xcfd67*, *Xwmc318*, *XBG313707*, and *Xgdm3*; 65°C for *Xbarc205*. Genetic maps were constructed using MAPMAKER/EXP3.0 (Lander et al. 1987).

Flowering data from the experiment to determine the interaction between vernalization and *Vrn-D4* alleles were analyzed using a 2 × 2 factorial ANOVA. A logarithmic transformation was used to improve the adjustment of the data to the ANOVA assumptions. Statistical analyses were performed using SAS version 9.1 (SAS Institute Inc. 2006).

Real-time quantitative PCR (Q-PCR)

Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was synthesized using the SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Q-PCR was performed on an ABI PRISM 7000 SDS (Applied Biosystems, Foster City, CA, USA) using SYBR® GREEN. PCR setup and reaction conditions were as reported before (Fu et al. 2007). The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to normalize and calibrate transcript values relative to the wheat translation elongation factor 1 alpha-subunit (*TEF1*, primers 5′-GCCCTCCTTGCTTTCACTCT-3′ and 5′-AACGCGCCTTTGAGTACTTG-3′, 99% efficiency). The quantitative RT-PCR SYBR® GREEN systems for *Vrn-1* (Yan et al. 2003), *Vrn-2* (Distelfeld et al. 2009b), and *Vrn-3* (Yan et al. 2006) genes have been published before.

Results

Differences between TDF stocks

Molecular markers for the *Vrn-A1* and *Vrn-B1* loci (Fu et al. 2005; Yan et al. 2004a) were used to confirm previous genetic studies suggesting that the original cultivar Triple Dirk has dominant *Vrn-A1* and *Vrn-B1* alleles (Stelmakh 1987b). The same markers demonstrated that the TDF-J stock carries the expected recessive *vrn-A1* and *vrn-B1* alleles for winter growth habit (Fig. 1a, b), but also showed that the TDF-US stock carries the dominant *Vrn-A1a* allele (140-bp insertion in the promoter region) and the dominant *Vrn-B1* allele (deletion in intron 1) (Fig. 1a, b). In addition, the TDF-US showed the same alleles for microsatellite markers *Xgwm190, Xcfd81,* and *Xbarc205* in the *Vrn-D4* region as TDC (same as original Triple Dirk cultivar), which were different in the TDF-J stock (Fig. 1c).

The absence of *Vrn-D4* in the TDF-US stock was confirmed in a population of 118 F_2 plants from the cross between TDF-US × CS($5D_{5402}$). Segregation for flowering time was associated with the *Vrn-A1* and *Vrn-B1* regions but no differences in flowering time were associated with marker *Xcfd67* from the *Vrn-D4* region (data not shown).

Taken together, the previous results suggest that the TDF-US stock is not the original TDF stock described by Pugsley (1972) and is, more likely, a contamination with the original Triple Dirk stock. Therefore, the TDF-US stock was discarded and all further analyses were performed using the TDF-J stock.

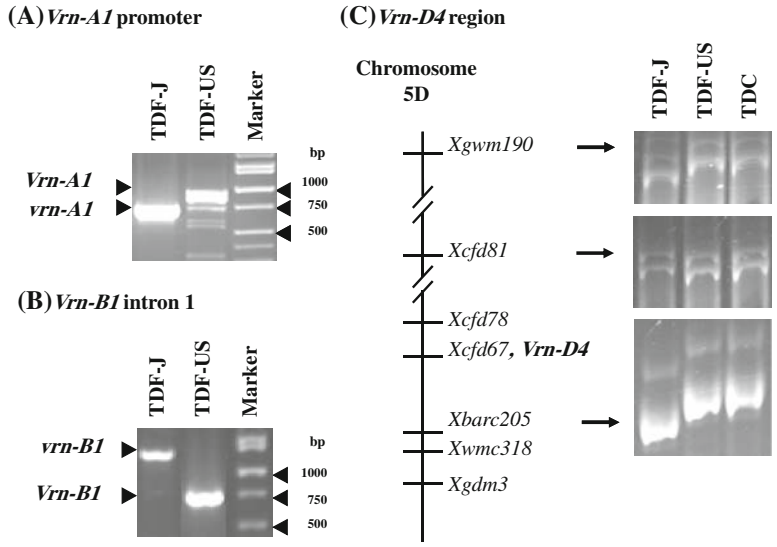

Fig. 1 Heterogeneity of TDF stocks. **a** PCR analysis of the *Vrn-A1* promoter. A 140 bp insertion is present in the *Vrn-A1* allele in TDF-US, and absent in the *vrn-A1* allele in TDF-J. **b** PCR analysis of *Vrn-B1* first intron. The *first lane* in the gel shows DNA from TDF-J amplified with primers F and R4 (Fu et al. 2005) that detect the absence of the first intron deletion (*vrn-B1* allele), and the *second lane* in the gel shows DNA from TDF-US amplified with the primers F and R3 (Fu et al. 2005) that detect the presence of the first intron deletion (*Vrn-B1* allele). **c** The TDF stocks have different haplotypes for markers in the *Vrn-D4* region. Three SSR markers *Xgwm190*, *Xcfd81*, and *Xbarc205* showed polymorphisms between TDF-J and TDF-US and no polymorphism between TDF-US and TDC

Effect of the duration of the vernalization treatment on flowering time in different Triple Dirk NILs

The comparison of the TDF-J with the Triple Dirk NILs for the *Vrn-1* dominant alleles revealed differences in the residual effect of vernalization on these alleles for spring growth habit. In the absence of vernalization, the dominant *Vrn-A1* allele (TDD) conferred the earliest flowering time and *Vrn-D4* was intermediate between *Vrn-B1* (TDB) and *Vrn-D1* (TDE). A factorial ANOVA including NILs and vernalization treatments as factors showed significant differences among lines ($P < 0.0001$) and among vernalization treatments ($P < 0.0001$). The presence of a significant interaction between NILs and vernalization treatments ($P < 0.0001$) indicated that the different NILs respond in different ways to vernalization treatments of different durations. Pair-wise comparisons among the four isogenic stocks using the Tukey test revealed significant differences for all comparisons ($P < 0.0001$). Highly significant differences among NILs ($P < 0.0001$) were also detected in the eight separate ANOVAs for each of the vernalization treatments.

TDD (*Vrn-A1*) showed no acceleration of flowering time for any of the vernalization treatments and was the earliest to flower for the 0 and 5 days vernalization treatments (Fig. 2). The TDB (*Vrn-B1*), TDE (*Vrn-D1*), and TDF-J (*Vrn-D4*) stocks showed a small residual response to vernalization that was satisfied after 25 days of vernalization (Fig. 2). The difference for TDF-J (*Vrn-D4*)

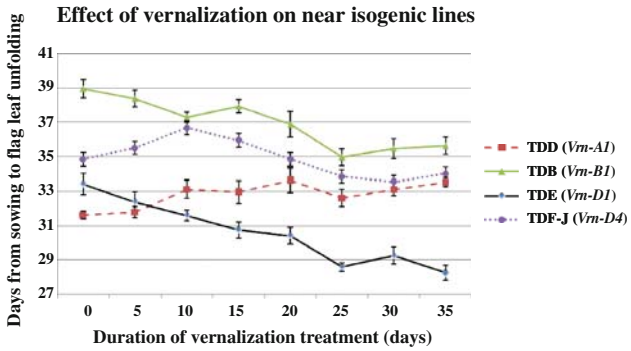

Fig. 2 Response of Triple Dirk near isogenic lines to vernalization treatments of different duration. *Error bars* are standard errors of the means

between the non-vernalized (0 days) and the average of the three saturating vernalization treatments (25, 30, and 35 days) was 1 day, but the difference was significant ($P = 0.009$). The acceleration of flowering in TDB (*Vrn-B1*) and TDE (*Vrn-D1*) was continuous from 5 to 25 vernalization days, but in TDF-J (*Vrn-D4*) no acceleration of flowering time was observed for the shorter vernalization treatments (5 and 10 days, Fig. 2). Although the profiles for TDF-J (*Vrn-D4*) and TDD (*Vrn-A1*) were similar for the 0, 5, 10, and 15 days, *Vrn-D4* was approximately 3 days later than *Vrn-A1* for each of these treatments. These results suggest that the response of *Vrn-D4* to vernalization might be different from the one observed for the dominant *Vrn-1* alleles.

Vrn-D4 mapping

The 144 F_2 plants from the cross TDF-J × Akakawaaka segregated into 111 spring-type plants and 33 winter-type plants (Fig. 3a), which fits a 3:1 ratio for a single dominant gene segregation ($\chi^2 = 0.33$, $P = 0.56$). In this population, molecular marker *Xcfd67* was found to cosegregate with the differences in flowering time (Fig. 3a). However, the low level of polymorphisms found between TDF-J and Akakawaaka precluded the development of a genetic map using this population.

A screen of additional Japanese winter cultivars showed that Hayakomugi was more polymorphic with TDF-J than Akakawaaka. Six microsatellite markers and an EST-derived marker were polymorphic in this TDF-J × Hayakomugi population. The frequency distribution of flowering times in this population was bimodal, but with a small overlap between *Xcfd67* classes (Fig. 3b). F_3 seeds from the F_2 plants with flowering times within the overlapping region as well as from some F_2 plants with critical recombination events in the *Vrn-D4* region were selected to perform progeny tests and provide a more accurate estimate of the original F_2 plants phenotype. All the plants with recombination events between flanking markers *Xcfd78* and *Xbarc205* showed clear flowering phenotypes (either in the F_2 or in the F_3 progeny tests), which facilitated a precise mapping of the *Vrn-D4* gene within this interval completely linked to *Xcfd67*. Using these additional data, the plants from this population were classified into 186 spring-type plants and 72 winter-type plants (Fig. 3b). This segregation fits a 3:1 ratio for a single dominant gene segregation ($\chi^2 = 1.16$, $P = 0.28$).

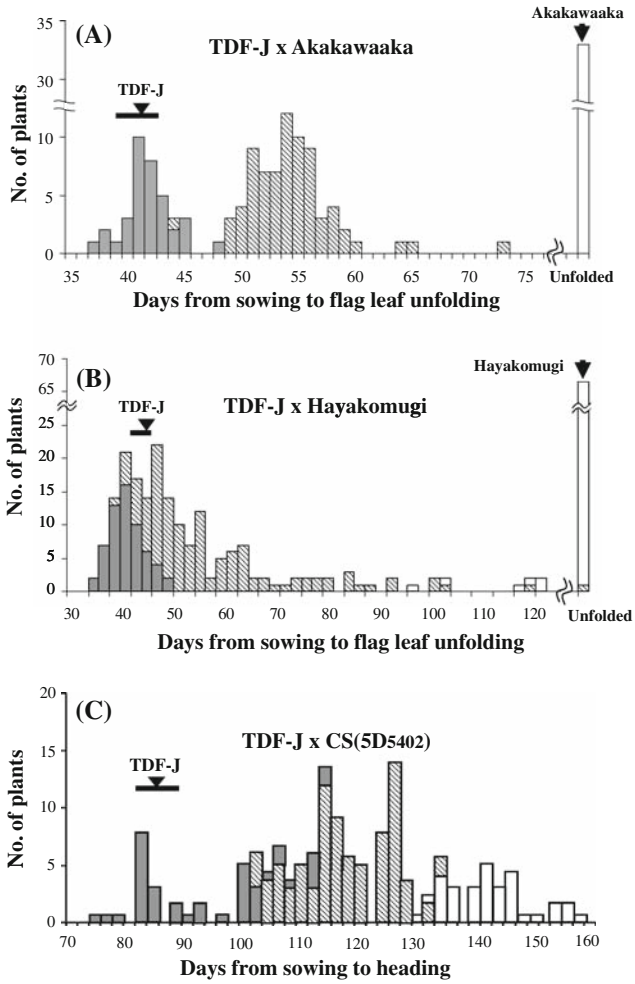

Fig. 3 Frequency distribution of the days from sowing to flag leaf unfolding (**a** and **b**) or ear emergence (**c**) in the F_2 populations derived from the crosses between TDF-J and **a** Akakawaaka, **b** Hayakomugi, and **c** CS($5D_{5402}$). Plants were grown under a non-vernalizing conditions (20°C) and long day photoperiod (**a** 24 h; **b**, **c** 16 h light). Plants were classified by their *Xcfd67* genotype (*Xcfd67* is linked to *Vrn-D4*) as follows: *gray rectangles* correspond to plants homozygous for TDF-J allele, *striped rectangles* to heterozygous plants, and *white rectangles* to plants homozygous for the *Xcfd67* allele from the other parent

The seven polymorphic markers were confirmed to be from chromosome 5D using the nulli-tetrasomic line missing that chromosome, and were assigned to different chromosome bins as described in Fig. 4a, b. Since *Vrn-D4* was completely linked with long arm marker *Xcfd67* and short arm marker *XBG313707*, it was not possible to establish its chromosome arm location. The three linked markers were mapped within a 1.8 cM region flanked by *Xcfd78* in the short arm and *Xbarc205* in the long arm (Fig. 4c).

In TDF-J × CS($5D_{5402}$) population, there was a clear association between the marker classes and flowering time, with a small number of ambiguous plants (Fig. 3c). However, since all the plants with recombination events between *Xcfd81* and *Xbarc143* showed unambiguous flowering phenotypes in the F_2 or F_3 progeny tests, it was possible to map the *Vrn-D4* completely linked to markers *Xcfd67*, *XBG313707*, *Xgdm68*, *Xbarc205*, and *Xgdm3* (Fig. 4c). If the genotype of the few plants with intermediate flowering times (and no recombination between flanking markers) is inferred based on the genotype of the *Vrn-D4* flanking markers, the 159 F_2 plants from the cross TDF-J × CS($5D_{5402}$) can be classified into 124 spring-type plants and 35 winter-type plants, which fits a 3:1 segregation ratio for a single dominant gene segregation ($\chi^2 = 0.76$, $P = 0.38$).

All markers that were polymorphic in the TDF-J × Hayakomugi population were also polymorphic in this population and were mapped. In addition, microsatellite marker *Xgdm68* not mapped on the previous population was added to this map. The TDF-J × CS($5D_{5402}$) population showed lower levels of recombination than the TDF-J × Hayakomugi population, which was reflected in smaller genetic distances (52% reduction) and lower resolution of the markers in the centromeric region. In this population, *Vrn-D4* was mapped completely linked to five

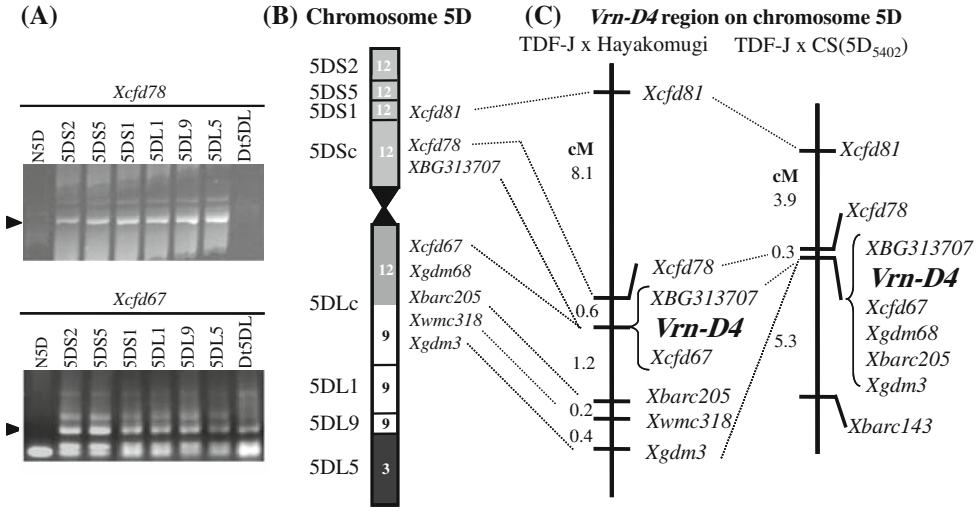

Fig. 4 Physical and genetic mapping of *Vrn-D4*. **a** Example of physical mapping of microsatellite markers *Xcfd78* and *Xcfd67* using cytogenetic stocks N5D (nulli-tetrasomic line missing chromosome 5D), Dt5DL (ditelosomic line missing the 5DS arm), and 5DS2 to 5DL5 (deletion lines for the short and long arm). **b** Assignment of markers to chromosome bins. The *numbers within each bin* indicate the colinear rice chromosome. **c** Genetic maps of *Vrn-D4* relative to molecular markers in the populations from the crosses TDF-J × Hayakomugi and TDF-J × CS($5D_{5402}$)

molecular markers flanked by *Xcfd78* in the short arm and *Xbarc143* in the long arm (Fig. 4c).

Interaction between *Vrn-D4* alleles and vernalization

A separate experiment using selected F_3 plants from the TDF-J × Hayakomugi population demonstrated significant interactions for flowering time between the *Vrn-D4* alleles and the presence or absence of vernalization treatment (2 × 2 factorial ANOVA, $P < 0.0001$). Significantly larger differences in heading time between *Vrn-D4* alleles were detected among unvernalized plants (35 days) than among vernalized plants (10 days). These data confirmed that vernalization modulates the effect of the *Vrn-D4* alleles on flowering time.

To see how other vernalization genes were affected by the *Vrn-D4* alleles, transcript levels of *Vrn-1*, *Vrn-2*, and *Vrn-3* were compared between TDF-J (*Vrn-D4* allele for spring growth habit) and CS($5D_{5402}$) (*vrn-D4* allele for winter growth habit) using quantitative RT-PCR. Plants from the two lines were sown at the same time in a greenhouse at non-vernalizing temperatures (20–25°C) under long day conditions (samples were taken at noon). At the time of leaf sample collection for RNA extraction, TDF-J plants were heading and CS($5D_{5402}$) plants were still at the vegetative stage. At this stage, TDF-J leaves showed higher transcript levels of the flowering promoting genes *Vrn-1* (>4,000-fold increase, $P = 0.0002$) and *Vrn-3* (>60,000-fold increase, $P = 0.006$) and reduced levels of the flowering repressor *Vrn-2* (>80-fold reduction, $P = 0.012$) than CS($5D_{5402}$) (Table 2).

Table 2 Transcript levels of *Vrn-1*, *Vrn-2*, and *Vrn-3* in lines with different *Vrn-4* alleles (normalized and calibrated)

Gene	Line[a]	Avg. $2^{-\Delta\Delta CT}$	SE	*P*
Vrn-1	TDF-J	12445	914	0.0002
	CS($5D_{5402}$)	3	1	
Vrn-2	TDF-J	6	4	0.012
	CS($5D_{5402}$)	483	108	
Vrn-3	TDF-J	241601	46035	0.006
	CS($5D_{5402}$)	4	1	

[a] TDF-J and CS($5D_{5402}$) carry *Vrn-D4* and *vrn-D4*, respectively

Discussion

Heterogeneity in TDF stocks

Pugsley (1972) identified the spring growth habit gene *Vrn-D4* in the cultivar Gabo and showed that it was not allelic to any of the *Vrn-1* homoeologs. Gabo was a major cultivar in Australia from the late 1940s to the late 1960s (O'Brien et al. 2001). Gabo's pedigree includes the Indian cultivar Muzaffar Nagar. Early-maturing forms were introduced from India to avoid rust and drought in Australian breeding programs (Lupton 1987). Since the allelic frequency of *Vrn-D4* is relatively high in India compared with other regions (Iwaki et al. 2000, 2001), it was assumed that Muzaffar Nagar might have been Gabo's donor of *Vrn-D4*. This hypothesis still needs experimental confirmation.

The *Vrn-D4* allele for early flowering from Gabo was transferred by Pugsley (1972) to Triple Dirk C by backcrossing. The resulting line with the dominant *Vrn-D4* allele and recessive alleles at all the other vernalization genes was designated TDF. However, several studies have questioned the existence of *Vrn-D4* or its chromosome location. Maystrenko (1980) suggested that Gabo has both *Vrn-B1* and *Vrn-D4* but erroneously assigned them to chromosomes 2B and 5B, respectively. Stelmakh (1987b) initially suggested that TDF and Gabo have both *Vrn-A1* and *Vrn-B1* but not *Vrn-D4*. This allelic combination is the same we found in the TDF-US stock and suggests the possibility that Stelmakh used a similar incorrect TDF stock. In his paper, Stelmakh mentioned that the seeds of TDF and Gabo he used were directly provided by Pugsley in 1981 and 1974, respectively. Later, Stelmakh (1998) conducted additional genetic analysis using populations from the cross between a TDF stock from Japan and *Vrn-1* tester lines and concluded that TDF-J has *Vrn-D4*, but that the TDF selection Y used in his 1987 paper had the *vrn-D4* allele for winter growth habit.

Gotoh (1979) conducted genetic analyses using a TDF stock provided to him by Pugsley before 1976 and confirmed the existence of *Vrn-D4* as a different gene, not allelic to any of the *Vrn-1* homoeologs. Kato et al. (2003) confirmed that *Vrn-D4* was linked to molecular marker *Xgdm3* in the centromeric region of chromosome 5D, and more than 50 cM proximal from the location of the *Vrn-D1* locus in the middle of the long arm (Kato et al. 2003). Goncharov (2003) used the same TDF-J and confirmed the existence of *Vrn-D4* in TDF and Gabo, although he failed to detect the 5D chromosome location, possibly because of a problem in his monosomic tester line Bersée mono 5D.

In summary, there seems to be some heterogeneity among different TDF stocks, which might be caused by contamination of the TDF seeds by the original Triple Dirk variety. The incorrect TDF stocks can now be readily identified using available molecular markers for *Vrn-A1* (Yan et al. 2004a) and *Vrn-B1* (Fu et al. 2005).

Vrn-D4 mapping

In this study, the *Vrn-D4* gene was mapped in the centromeric region of chromosome 5D, which was consistent with preliminary mapping data generated by Kato et al. (2003). The collinear region in rice chromosome 12 includes several flowering QTLs (Mei et al. 2003; Nagata et al. 2002; Septiningsih et al. 2003; Uga et al. 2007). However, it is currently not possible to determine whether *Vrn-D4* corresponds to any of these rice QTL, because the arm location of *Vrn-D4* in wheat is not yet known, and therefore, the colinear region in rice chromosome 12 is too large. We are currently expanding the mapping population to generate additional recombination events to delimit better the chromosome location of *Vrn-D4* in wheat and its collinear region in rice. Additional sequenced-based markers (such as BG313707) will also be necessary to establish a better correspondence between the two regions.

In the TDF-J × CS($5D_{5402}$) population, genetic distances were 2.5-fold smaller than in the TDF-J × Hayakomugi population (Fig. 4). This might be attributed to the high level of polymorphisms detected between chromosomes 5D from CS($5D_{5402}$) and from hexaploid wheat. These results are in agreement with previous studies that showed a lower chiasma formation at metaphase I between homologous chromosomes from divergent varieties compared with identical chromosomes from the same variety (Dvorak and McGuire 1981). Particularly relevant to this study is the significant decrease in chromosome pairing detected between chromosome 5D from Chinese Spring and chromosome 5D from *Ae. tauschii* in a Chinese Spring genetic background relative to the pairing of identical 5D chromosomes (Dvorak 1988).

In summary, a combination of multiple mapping populations, one maximizing recombination and the other one maximizing polymorphisms, seems to be the best strategy to accelerate the development of a high density map of the *Vrn-D4* gene. The TDF-J × CS($5D_{5402}$) population can be used first to select the closest markers to *Vrn-D4*, and then, the efforts to find polymorphisms in the TDF-J × Hayakomugi population can be focused in a reduced number of selected markers.

Effect of *Vrn-D4* on vernalization response

This study has confirmed the existence of a single locus for early flowering in all three crossing populations between TDF-J and winter lines, and demonstrated that the effect of this gene on flowering time is modulated by vernalization requirement. The significant interaction detected between *Vrn-D4* alleles and vernalization is a hallmark of genes that are part of the vernalization pathway. The higher transcript levels of *Vrn-1* and *Vrn-3* and lower transcript level of *Vrn-2* in TDF-J (*Vrn-D4* allele) relative to CS($5D_{5402}$) (*vrn-D4* allele) planted at the same time suggest that *Vrn-D4* acts upstream (or is part of) the feedback regulatory loop formed by *Vrn-1*, *Vrn-2*, and *Vrn-3* (Distelfeld et al. 2009a).

The comparison of the vernalization response of the different Triple Dirk NILs showed that the *Vrn-D4* allele for spring growth habit has a residual vernalization response, a phenomenon also observed for the *Vrn-B1* and *Vrn-D1* alleles, both here and in previous studies (Berry et al. 1980; Pugsley 1972). However, the responses of these two last genes differed slightly from the one observed for *Vrn-D4*, particularly for plants exposed to short vernalization periods (5–10 days). Flowering in plants carrying the

Vrn-B1 and *Vrn-D1* alleles was accelerated by 5–10 days exposures to cold temperatures, but no acceleration was detected for *Vrn-D4* for similar treatments. These differences may reflect separate roles of these genes in the vernalization pathway, but a final answer to this question will require the cloning of the *Vrn-D4* gene.

Spring growth habit gene *Vrn-D4* for wheat improvement

Vrn-D4 has not been extensively used in spring wheat breeding programs in North America, Europe, and East Asia including Japan (Goncharov 1998; Gotoh 1979). In Europe and North America, *Vrn-A1* and *Vrn-B1* are predominant, while in Asia, especially in Japan, *Vrn-D1* is frequently found (Goncharov 1998; Gotoh 1979; Stelmakh 1987a). The *Vrn-D1* allele is frequent in fall-planted spring wheats, whereas the stronger *Vrn-A1* allele is present in high frequency among spring-planted spring varieties (Fu et al. 2005; Iqbal et al. 2007; Iwaki et al. 2000, 2001; Zhang et al. 2008).

Seki et al. (2007) analyzed the effects of *Vrn* genes on the timing of transition to adult phase using Abukumawase NILs and found that in fully vernalized plants grown in the field the NILs with the *Vrn-D4* and *Vrn-A1* alleles were earlier than those with the *Vrn-B1* and *Vrn-D1* alleles. In addition, the results presented here suggest that the *Vrn-D4* gene differs from *Vrn-B1* and *Vrn-D1* in its response to short cold intervals (Fig. 2). A strong *Vrn-D4* allele has been reported in the Italian cultivar Mara (Worland et al. 1987), which suggests that there might be multiple alleles of *Vrn-D4* with different effects on flowering time. In summary, these results indicate that the *Vrn-D4* gene might be useful for fine tuning heading time and vernalization requirement in hexaploid wheat.

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