Molecular Genetic Study of Earliness-related Genes in Wheat

Tetsuya Yoshida
Fukukaen Nursery & Bulb Co.Ltd

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CHAPTER I.
Introduction

Wheat (*Triticum aestivum* L.) is one of the most important crops in the world and has the largest cultivated area among crops. By reflecting such situations, wheat is widely grown in quite different conditions including severe cold temperature during winter at higher latitude regions like North Europe and Siberia and subtropical climate at low latitude regions near equator, although it is mainly grown in relatively warm region including Asia, North America, and Europe (Stelmakh 1990).

The Japanese Archipelago lies from North to South. Because of its wide range of latitude, it belongs to various climate zones: the subarctic zone, the temperate zone, and the tropical zone. Therefore, heading traits of wheat varieties are markedly different depending on local climates they adapted to (Wada and Akihama 1934). Although wheat is generally sown in autumn in Japan, growth habit of varieties is different depending on the regions. Winter type is grown in cold area including most part of Hokkaido and Tohoku region, while spring type is grown in south-western warm region which covers western part of Japan from Kanto to Kyusyu. The only exception is eastern part of Hokkaido where spring type "Haruyokoi" is the principal variety and is grown by spring sowing cropping.

In Japan, grain maturation and harvesting time of intermediate- and late-heading wheat often encounter rainy season around June. Intermittent rain during the rainy season causes several problems including pre-harvest sprouting, diseases like Fusarium head blight, and immature grain. Even when pre-harvest sprouting does not occur, overhydration within grain activates proteases and enzymes causing starch degradation, and it results in low pasting viscosity properties and secondary processing suitability (Nagao 1996; Noda 1999). Furthermore, it also causes change of pericarp color which results in decrease of commercial value. It is well known that pre-harvest sprouting is associated with seed dormancy and pericarp color. Generally, Japanese landraces have brown grain which has red pericarp and is considered to be associated with tolerance to pre-harvest sprouting. In fact, Japanese landraces are known to have tolerance.

Among Japanese varieties, "Zenkojikomugi" has the strongest tolerance against pre-harvest sprouting which is known to be controlled by several genes (Toda et al. 1969). Because it is not feasible to accumulate all of these genes as well as those for other traits like grain quality, high quality variety with strong tolerance comparable to "Zenkojikomugi" has never been developed so far. Alternative solution instead of

reinforcing the tolerance is the development of extremely early varieties because it makes maturity and harvesting ealier and can prevent them from encountering rainy season. In fact, several extremely earliy varieties like “Abukumawase” and “Fukuwasekomugi” have already been developed. However they are spring-type wheat and their earliness is conferred by reducing photoperiod sensitivity. Such varieties differentiate ear primordia and start internode elongation very early, so they are very likely to suffer frost injury including death of ear primordia in case of early sowing or warmer winter which often occurs in recent years (Goto 1975). To avoid frost injury, application of winter-type variety with extremely early heading is considered to be effective. Such varieties e.g. “Iwainodaichi” (Taya et al. 2003) and “Satonosora” (Takahashi et al. 2010) have already been developed and they have been cultivated in Kyushu and Kanto regions respectively.

Heading time of wheat is a complex character comprised of three genetic factors: vernalization requirement, photoperiodic response, and earliness *per se*. Earliness *per se*, different from the other two, is independent of environmental factors and is recognized as the earliness by nature which is specific to varieties. This character is controlled by several minor genes (Kato and Wada 1999, Ohara 2001) and they were assigned to different chromosomes: Miura and Worland (1994) reported a gene on chromosome 3A, Kato et al.(1999) reported a gene on chromosome 5A, and Hoogendoorn (1985) reported genes on chromosomes 3A, 4A, 4D, 6B, and 7D. On the contrary, vernalization requirement and photoperiodic response depend on environmental factors and they ensure safer heading (reproduction) by delaying heading time until environmental condition becomes favorable.

Vernalization requirement is controlled by four different genes, *Vrn-1*, *Vrn-2*, *Vrn-3*, and *Vrn-4* (Flood and Hallolan 1986, Worland et al. 1987). Among them, *Vrn-1* plays most important role in adaptation to regions with different climate conditions including cooler, warmer, and spring-sowing regions. Natural variation is known for all of three *Vrn-1* homoeologs. Most of the spring-type wheat including landraces as well as improved varieties carries spring allele(s) for either *Vrn-1* homoeologs (Iwaki et al. 2000; 2001). As for *Vrn-2*, natural variation is known only in einkorn wheat (*T. monococcum* L.) and barley (*Hordum vulgare* L.) and it has never been found in hexaploid wheat which carries the winter allele (Yan et al. 2004). Among four *Vrn* genes, *Vrn-2* is the only gene whose recessive allele confers spring growth habit while dominant allele confers winter growth habit. On the contrary, dominant allele confers spring growth habit in the other three genes. Natural variation for *Vrn-3* has been found only in B genome and a variety “Hope” carries the spring allele *Vrn-B3* on

chromosome 7B (Yan et al. 2006). As for *Vrn-4*, natural variation has been found only in D genome. Kato et al. (2003) reported that a wheat line "Triple Dirk (F)" carries the spring allele *Vrn-D4* on chromosome 5D.

By the recent progress in the molecular genetic study on vernalization requirement, all *Vrn* genes except *Vrn-4* have already been cloned via positional cloning strategy and their function has gradually been disclosed (Yan et al. 2003; Yan et al. 2004b; Yan et al. 2006). *Vrn-1* encodes a MADS-box transcription factor similar to Arabidopsis *AP1/FRUITFULL* gene family which plays a crucial role in transition from vegetative growth to reproductive growth. *Vrn-2* encodes a transcription factor with zinc finger domain and CCT domain. *Vrn-3* (formerly known as *Vrn5* or *Vrn-B4*) encodes an ortholog of Arabidopsis flowering promoter *FT* (Yan et al. 2006). In winter wheat, *Vrn-2* functions as a repressor of *Vrn-1* until vernalization requirement is satisfied. As wheat is vernalized by cold temperature and short photoperiod conditions, *Vrn-2* is gradually down-regulated. This results in the up-regulation of *Vrn-1* and *Vrn-3* by which promotion of floral development occurs (Hemming et al. 2008; Yan et al. 2006). *Vrn-3* is up-regulated under long photoperiod conditions and it up-regulates *Vrn-1*. However, the genetic mechanism of vernalization requirement still remain unknown in detail. To disclose this machanism, further analysis on all genes involving this mechanism is important. As the initial step, molecular cloning of *Vrn-D4* will be required.

Photoperiodic response is the most important factor that determines heading time of wheat (Yasuda and Shimoyama 1965, Kato and Yamagata 1988). Wheat is a long day plant. In photoperiod-sensitive wheat, flowering initiation is delayed under short photoperiod during winter and it is promoted under long photoperiod, while such delay does not occur under short photoperiod in photoperiod-insensitive wheat. This mechanism ensures the safer reproduction by delaying reproductive growth until the environmental condition becomes desirable. Photoperiodic response is controlled by three homoeologs *Ppd-A1*, *Ppd-B1*, and *Ppd-D1* on chromosomes 2A, 2B, and 2D respectively (Keim et al. 1973; Pirasteh and Welsh 1975; Law et al. 1978; Scarth and Law 1983). Dominant alleles for these genes confer photoperiod-insensitivity which results in flower initiation even under non-inductive short photoperiod, while recessive alleles confer photoperiod-sensitivity. Recently, Beales et al. (2007) cloned three *Ppd-1* homoeologs and found a 2089bp deletion in promoter region of the photoperiod-insensitive allele *Ppd-D1a* which did not exist in the photoperiod-sensitive allele *Ppd-D1b*. Their result suggested that the photoperiod-insensitive allele occurred by the mutation (deletion) at this region of the photoperiod-sensitive allele. On the contrary, such mutation has not been detected in photoperiod-insensitive alleles for

Ppd-A1 and *Ppd-B1*.

The objective of this study is to elucidate heading time genes in detail. In chapter 2, fine mapping of the vernalization requirement gene *Vrn-D4* was described. Fine mapping was conducted as the initial step for molecular cloning and as the result, DNA markers cosegregating with *Vrn-D4* were detected. In chapter 3, world-first finding of a wheat variety carrying the photoperiod-insensitive allele for *Ppd-A1* was described. It was also described that there was sequence variation in the photoperiod-insensitive alleles for *Ppd-A1* and *Ppd-B1* that could explain the difference of effect between photoperiod-sensitive and –insensitive alleles.

CHAPTER Ⅱ.

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

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).

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.

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	Japansese winter variety
Hayakomugi	*vrn-D4*	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	Japansese winter variety
CS($5D_{5402}$)	*vrn-D4*	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	Chinese Spring substitution line with *Ae. Tauschii* 5D chomosome

[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

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/m²/s. Planting density was one plant per 2.8 × 4.3 cm² 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 cm².

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′-CACCACCAGTAACCCAGCC-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.

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*) 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 ($\chi^2 = 1.16$, $P = 0.28$).

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 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-D4* alleles (normalized and calibrated)

Gene	Line[a]	Avg. $2^{-\Delta\Delta CT}$	SE	*P*
Vrn-1	TDF-J	12445	94	0.0002
	CS($5D_{5402}$)	3	1	
Vrn-2	TDF-J	6	4	0.012
	CS($5D_{5402}$)	483	108	
Vrn-3	TDF-J	242601	46035	0.006
	CS($5D_{5402}$)	4	1	

[a] TDF-J and CS(5D5402) carry *Vrn-D4* and *vrn-D4*, respectively

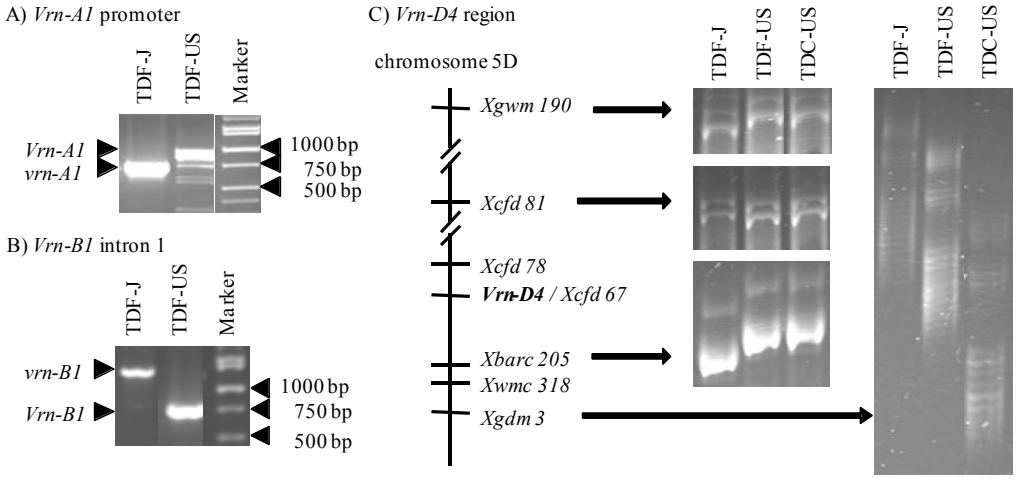

Fig. 1 Heterogeneity of TDF stocks.
a PCR analysis of the *Vrn-A1* promoter. A 140 bp insertion is present in the *Vrn-A1* allele in TDF-US, and absent in the *Vrn-A1* allele in TDF-J. b PCR analysis of *Vrn-B1* first intron. The first lane in the gel shows DNA from TDF-J amplified with primers F and R4 (Fu et al. 2005) that detect in 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*, *Xbarc205* showed polymorphisms between TDF-J and TDF-US and no polymorphism between TDF-US and TDC.

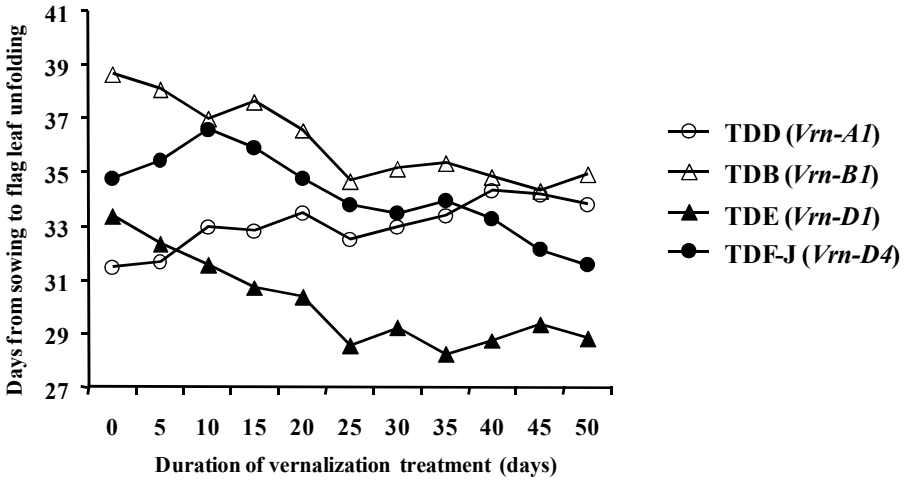

Fig. 2 Response to Triple Dirk near isogenic lines to vernalization treatments of different duration.

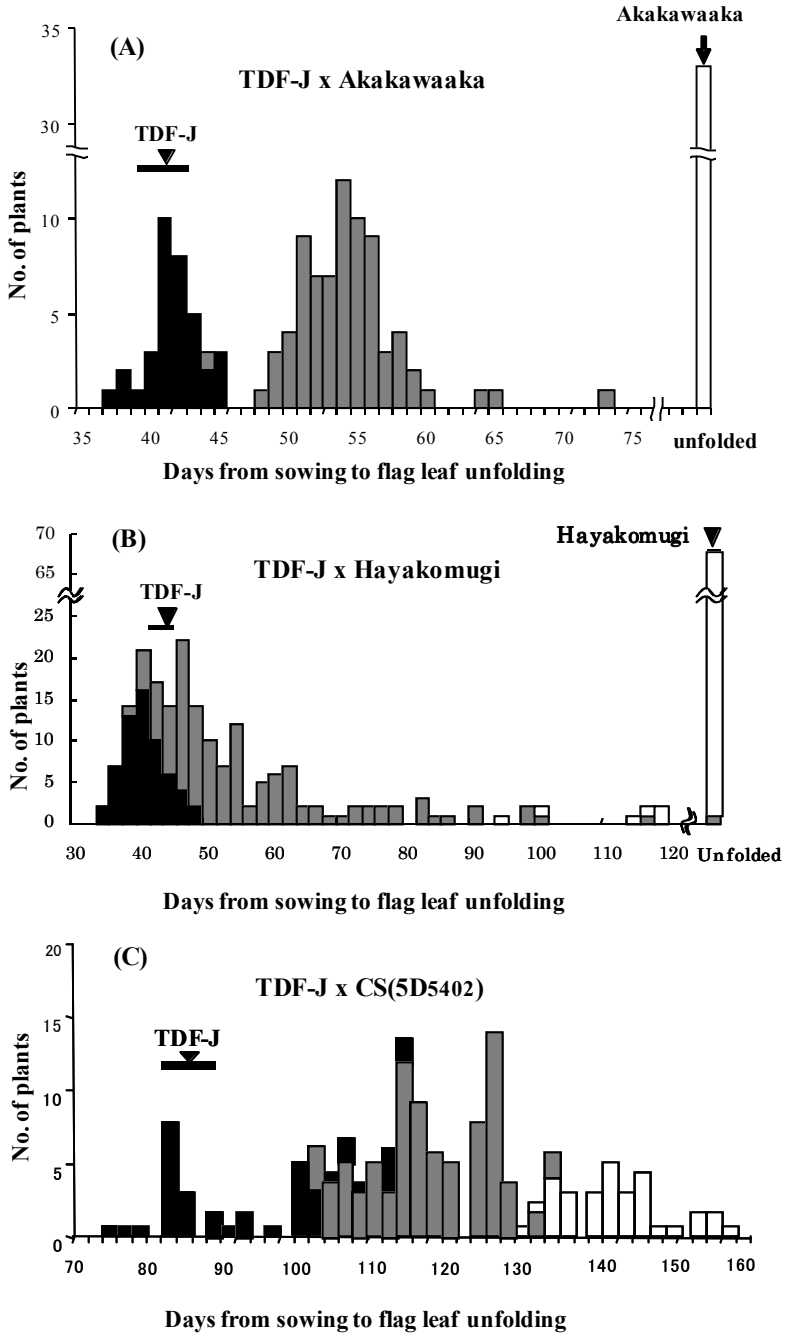

Fig. 3 Frequency distribution of the days from sowing to flag leaf unfolding (a and b) or ear emergence (c) in the F_2 populations derived from the crosses between TDF-J and a Akaakwaaka, b Hayakomugi and c CS($5D_{5402}$). Plants were grown under a non-vernalizing conditions (20℃) and long day photoperiod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

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.

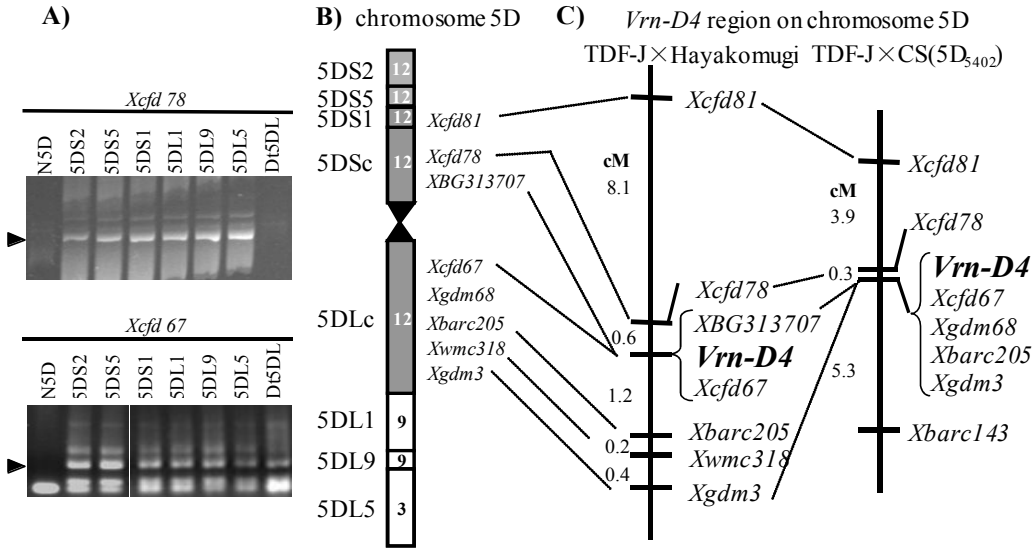

Fig. 4 Physical and genetic mapping of *Vrn-D4.*
a Example of physical mapping of microsatellite markers *Xcfd78* and *Xcfd67* using cytogenetic stocks N5D (nulli-tetrasomic line missing chromosome 5D), Dt5DL (ditelosomic line missing the 5DS arm), and 5DS2 to 5DL5 (deletion lines for the short and long arm). b Assignment of markers to chromosome bin. The *numbers within each bin* indicate the collinear 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($5D_{5402}$)

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.

CHAPTER Ⅲ.

Structural Variation in 5' Upstream Region of Photoperiod-Insensitive Alleles *Ppd-A1a* and *Ppd-B1a* Identified in Hexaploid Wheat (*Triticum aestivum* L.), and Their Effect on Heading Time

Introduction

Winter wheat is sown in autumn and harvested in early summer. Grain yield and quality are directly affected by various kinds of abiotic stresses encountered during grain filling stage, and largely reduced by drought and high temperature (Worland 1996). In monsoon climate area, on the contrary, intermittent rain during rainy season often causes pre-harvest sprouting and poor grain quality (Kato et al. 2001). It is important to avoid such stresses for ensuring stable wheat production, and enormous efforts have been made to breed early heading cultivars in many countries (Lupton 1987; Hoshino et al. 2000; Nam and Kim 2000). However, too early heading often results in the frost injury during winter and the sterility caused by low temperature at flowering stage (Marcellos and Single 1984; Chakrabarti et al. 2011). Therefore, heading time of wheat should be properly adjusted to respective growing conditions, in order to achieve the maximum grain yield.

Heading time of wheat is a complex character determined by three factors, that is, earliness *per se*, photoperiodic response and vernalization requirement (Yasuda and Shimoyama 1965; Kato and Yamagata 1988). Photoperiodic response proved to be the most important factor for the control of heading time of winter wheat grown in middle latitude areas, by correlation analysis using 158 wheat landraces with diverse geographical origin (Kato and Yamashita 1991). In the long-day plant wheat, heading is accelerated by long photoperiod, while it is delayed by short photoperiod in photoperiod-sensitive cultivars (Klaimi and Qualset 1973). The extent of photoperiod sensitivity, expressed as the difference of heading time under long and short photoperiods, depends on the genotype concerning this character (Klaimi and Qualset 1973), and insensitivity to photoperiod is a dominant character (Pugsley 1965). Genetic studies using the aneuplod lines revealed that insensitivity to photoperiod is determined by two genes located on chromosome 2BS and 2DS, which are designated as *Ppd-B1* (the former *Ppd2*) and *Ppd-D1* (the former *Ppd1*), respectively (Welsh et al. 1973; Keim et al. 1973; Pirasteh and Welsh 1975; Scarth and Law 1983; Scarth and Law 1984; Snape et al. 2001). Recent genetic studies of the Japanese wheat cultivars made it

clear that most of the intermediate heading cultivars represented by "Norin 61" carry a photoperiod-insensitive allele *Ppd-D1a*, while early heading cultivars carry a photoperiod-insensitive allele of *Ppd-B1* as well as *Ppd-D1a* (Tanio et al. 2005). In mid- and south-Europe, *Ppd-D1a* has been introduced for early wheat breeding to avoid high temperature in early summer (Worland 1996). These reports clearly indicated the importance of *Ppd-1* homoeologs for the adaptation to the growing condition in these regions.

Beales et al. (2007) cloned all three *Ppd-1* homoeologs and showed that they encode Pseudo-Response Regulators (*PRR*). The rice ortholog of *Ppd-1* is considered to be *Hd2* located on the distal end of rice chromosome 7L (Dunford et al. 2002; Murakami et al. 2005). In Arabidopsis, *PRR* genes are known to form a small family including five members, *PRR1*, *PRR3*, *PRR5*, *PRR7*, and *PRR9*. They are closely related to the circadian clock: *PRR1*, the same as *TOC1* (*TIMING OF CAB EXPRESSION 1*), forms the main feedback loop with two MYB transcription factors, *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*) and *LHY* (*LONG ELONGATED HYPOCOTYL*) (Nakamichi et al. 2010). *PRR5*, *PRR7*, and *PRR9* also form secondary loop in which they suppress *CCA1* and *LHY*. According to Beales et al. (2007), wheat Ppd-1 protein is most similar to PRR7 among Arabidopsis PRRs, suggesting that *Ppd-1* also plays a central part in wheat circadian clock.

Beales et al. (2007) explored 5' upstream region as well as exons and introns to find sequence variations that can explain difference between photoperiod-sensitive and –insensitive alleles of *Ppd-1* homoeologs. They found a 2089bp deletion in the 5' upstream region of *Ppd-D1a* that is absent in *Ppd-D1b*. This region seems to include sequences controlling *Ppd-D1* expression and presence/absence of the deletion is consistent with expression patterns. In contrast to the diurnal expression pattern of *Ppd-D1b*, *Ppd-D1a* does not show such fluctuation. As for *Ppd-A1*, two types of insensitive alleles of *Ppd-A1*, which had the different size of deletions in the 5' upstream region, were found in tetraploid wheat (Wilhelm et al. 2009). Both of the deletions occurred inside of the corresponding region of *Ppd-D1a* deletion, and their common approximately 900 bp region is believed to include critical sequence controlling gene expression. In fact, there is an approximately 100 bp region that is present about 120 bp upstream of transcription initiation site, which is highly conserved among some plant species such as wheat, barley, rice, and *Brachypodium*, although any important motifs like cis-element are not identified in this sequence.

As for *Ppd-A1* and *Ppd-B1* of common wheat, cultivars "C591" and "Chinese Spring" are listed as the carrier of insensitive allele *Ppd-A1a* and *Ppd-B1a*, respectively

(Catalogue of gene symbols for wheat, http://www.shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp). However, Mohler et al. (2004) reported that "C591" actually had *Ppd-B1a* instead of *Ppd-A1a*. Since "C591" was only one accession supposed to have *Ppd-A1a* in common wheat, it was required to screen further wheat accessions to identify a *Ppd-A1a* allele. The situation of *Ppd-B1a* was different, and several cultivars such as "Chinese Spring" and "Timstein" are known as its carrier through the analysis of inter-varietal chromosome substitution lines (Scarth and Law 1983, Scarth and Law 1984). However, unfortunately, Beales et al. (2007) could not detect the critical sequence polymorphism between *Ppd-B1a* and *Ppd-B1b* alleles. On the other hand, in our earlier work (Tanio et al. 2005), it was clearly shown that a Japanese early cultivar "Fukuwasekomugi" had *Ppd-B1a* as well as *Ppd-D1a*.

In the present paper, we aimed to describe the result of segregation analysis of three photoperiodic response genes *Ppd-A1*, *Ppd-B1*, and *Ppd-D1*, using a doubled haploid population derived from the cross between winter type NIL of Abukumawase and Chihokukomugi. The *Ppd-1* genotype of parental lines was determined by using linked SSR markers. Then, sequence polymorphism between sensitive and insensitive alleles of *Ppd-A1*, *Ppd-B1*, and *Ppd-D1* was determined to identify causal allelic variation, and a full set of DNA markers applicable to marker assisted selection of *Ppd-1* alleles was developed.

Materials and methods

Plant materials

A total of 80 doubled haploid (DH) lines derived from a winter wheat cross between two Japanese cultivars were developed by maize pollination. To develop one of the parental lines, winter type NIL of "Abukumawase" (referred as "Winter-Abukumawase"), "Abukumawase", an early cultivar with spring growth habit adapted to the southwestern part of Japan, was backcrossed five times to "Ebisukomugi" (the donor of winter growth habit) (Fujita et al. 1995). Another parental line "Chihokukomugi" was a winter type cultivar that used to be grown in Hokkaido, Japan. A photoperiod insensitive cultivar "Chinese Spring" carrying *Ppd-B1a* (Scarth and Law 1983) and a sensitive cultivar "Haruhikari" (Tanio et al. 2005) were also used for sequence comparison and marker development of *Ppd-1* genes.

Evaluation of heading date in the field

Heading date of DH lines and their parental lines was recorded per plant, using the materials sown in the experimental field of Okayama University (34° 41'N, 133° 55'E, 4m above sea level) on 21st November, 2001. Five plants were grown for each line with three replications.

DNA extraction

Total DNA was extracted from leaf blade of field grown plants independently from two plants for each line, by CTAB method (Murray and Thompson 1980) with minor modifications.

Estimation of *Ppd-1* genotype by the analysis of linked SSR markers

Heading date was employed to estimate photoperiodic response of DH lines, since photoperiodic response is the major determinant of heading time in the field as already mentioned. Based on the heading date, the earliest nine DH lines and the latest nine DH lines were selected for bulk segregant analysis using SSR markers, in order to estimate the *Ppd-1* genotype of parental lines. The SSR markers analyzed were

Xwmc177 (2AS), *Xgwm148* (2BS), and *Xgwm484* (2DS). The latter two markers are known to locate near *Ppd-1* homoeologous loci (Hanocq et al. 2007) and *Xwmc177* was selected based on the collinearity among homoeologous group 2 chromosomes.

PCR amplifications were performed in a 10μl volume containing 1μl 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, 0.5μM of primer, and 50 ng of template DNA. Amplification reactions were performed using i-Cycler (Bio-Rad, Hercules, CA, USA). 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°C to 60°C annealing (depending on marker) for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. Annealing temperature was 50°C for *Xgwm359* and *Xbarc297*, and 60°C for other five markers. The PCR products were electrophoresed on an 18% nondenatured polyacrylamide gel at a constant voltage of 240 V. Gels were stained with ethidium bromide and visualized by illumination with UV light.

Cloning and sequence analysis of *Ppd-1* homoeologs

To amplify the genomic sequence of *Ppd-1* homoeologs including 5' flanking sequence, primers specific to the 5' flanking region and 3' UTR sequence were designed for each gene, based on the wheat *Ppd-1* sequences reported by Beales et al. (2007). The detail of each primer is shown in Table 5; TaPpd-A1proF3 and TaPpd-1-3'UTRR3 for *Ppd-A1*, TaPpd-B1proF1 and TaPpd-B1-3'UTRR1 for *Ppd-B1*, and TaPpd-D1-proF3 and TaPpd-1-3'UTRR4 for *Ppd-D1*. The expected sizes of PCR amplicons were approximately 6.6 kb, 4.2 kb and 6.8 kb for *Ppd-A1*, *Ppd-B1* and *Ppd-D1*, respectively.

PCR amplifications were performed in a 10 μl volume containing 1μl of 1×Phusion™ GC Buffer, 0.3μl of DMSO, 0.2 units of Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Finland), 0.2 mM of dNTP, 0.2μM of each primer, and 150 ng of template DNA. Amplification reactions were performed using i-Cycler (Bio-Rad, Hercules, CA, USA). It was programmed to start with heating to 99°C for 3 min, followed by touchdown PCR, i.e., denature at 99°C for 10 sec, annealing at 70°C for 30 sec and extension at 72°C for 4 min for the first PCR cycle; thereafter, a decrease of 1°C for the annealing temperature in every cycle until 50°C was reached. Finally, 19 thermal cycles with 99°C for 10 sec, 50°C for 30 sec and 72°C for 4 min were performed. For the amplification of *Ppd-D1*, annealing temperature was modified, being from 65°C to 45°C for touchdown PCR and 45°C for the remaining 19 cycles. The PCR fragments were gel purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA), and then

cloned into pCR® 2.1-TOPO® Vector (Invitrogen, USA). Three clones of each gene from each parental line were sequenced by using M13 primers and ten primers listed in Table 5 to cover the whole sequence. Sequencing was conducted by PRISM® 3730 DNA Analyzer (Applied Biosystems).

Ppd-1 alleles are designated as follows, considering the phenotype with primary importance and their nucleotide sequence; the "a" suffix indicates a dominant photoperiod insensitive allele (*Ppd-A1a* etc.), while "b" indicates a photoperiod sensitive allele (*Ppd-A1b* etc.). Sequence variants of each allele are distinguished by the suffix like "*a.1*", "*a.2*" etc., with "*.1*" as the original in hexaploid wheat.

GenBank sequence accessions are; *Ppd-A1* "Winter-Abukumawase" AB646972, "Chihokukomugi" AB646973. *Ppd-B1* "Winter-Abukumawase" AB646974, "Chihokukomugi" AB646975. *Ppd-D1* "Winter-Abukumawase" AB646976, "Chihokukomugi" AB646977.

PCR markers to detect structural variation and genotyping of DH lines

To determine the *Ppd-1* genotype of 80 DH lines, PCR primer sets to detect the deletion of 1085 bp in 5' upstream sequence of *Ppd-A1* and the insertion of 308 bp in 5' upstream sequence of *Ppd-B1* were designed (Table 5). TaPpd-A1prodelF1 , TaPpd-A1prodelR3 and TaPpd-A1prodelR2 were used for *Ppd-A1*, TaPpd-B1proinF1 and TaPpd-B1proinR1 for *Ppd-B1*. Three primers, Ppd-D1-F1 , Ppd-D1-R1 and Ppd-D1-R2 developed by Beales et al. (2007) were used to detect the deletion of 2089 bp in 5' upstream sequence of *Ppd-D1*.

PCR amplification was done in a 10μl mixture containing 50 ng genomic DNA, 1 μl PCR buffer (Sigma, USA: 10 mM Tris-HCl (pH 8.3), 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer and 0.5 U *Taq* polymerase (Sigma, USA). Amplification reactions were carried out using i-Cycler (Bio-Rad, USA). The PCR cycle for the analysis of *Ppd-A1* was as follows: an initial denaturing step at 96ºC for 3 min, 35 PCR cycles at 96ºC for 30 sec, 57ºC for 30 sec, and 72ºC for 1 min. The final extension step was at 72ºC for 5 min. For *Ppd-D1*, the PCR cycle was modified as follows; annealing at 54ºC for 1 min and extension at 72ºC for 90 sec. Touchdown PCR was employed for *Ppd-B1*, i.e., annealing at 70°C for 30 sec for the first PCR cycle, and thereafter, a decrease of 1°C for the annealing temperature in every cycle until 60°C was reached. Finally, 29 thermal cycles with annealing at 60°C were performed. PCR products were electrophoresed on 1.5% agarose gel (GenePure LE, BM Bio, Japan) at a constant voltage of 100 V by using a horizontal gel electrophoresis system (Mupid-2,

Cosmo Bio, Japan). Gels were stained with Ethidium bromide and visualized by illumination with UV light.

Table 3 The detail of PCR primers used in this study.

Primers	Locus	Region	Sequence (5' → 3')	Annealing temperature[a]	Expected size
Primers to amplify whole sequence of *Ppd-1* homoeologs					
TaPpd-A1proF3	*Ppd-A1*	5' UTR	TTTGCAAACATGGTGAAAGA	70°C, 50°C	4327 bp (*Ppd-A1a*)
TaPpd-1-3'UTRR3		3' UTR	TGAGACGAGATGCATGAGGA		5412 bp (*Ppd-A1b*)
TaPpd-B1proF1	*Ppd-B1*	5' UTR	ACACTAGGGCTGGTCGAAGA	70°C, 50°C	4533 bp (*Ppd-B1a*)
TaPpd-B1-3'UTRR1		3' UTR	CCAGGAGATGAGACGAGATGA		4225 bp (*Ppd-B1b*)
TaPpd-D1-proF3	*Ppd-D1*	5' UTR	GCAGCTTTGGACATTTAGCTC	65°C, 45°C	4660 bp (*Ppd-D1a*)
TaPpd-1-3'UTRR4		3' UTR	TGAGACGAGATGCATGAGGA		6748 bp (*Ppd-D1b*)
Sequencing primers					
TaPpd-1-seqF5	*Ppd-1*	5' UTR	AACATCCTAGTGGCTCACG		
TaPpd-1-seqF4		5' UTR	TGAACCAACAAACTTGATCC		
TaPpd-1-seqF3		5' UTR	ATAGGTTGAAAGATTACCAACA		
TaPpd-1-seqF2		5' UTR	GGGCCCACAAAATCCACA		
TaPpd-1-seqF1		5' UTR	CGATTGGGGATCGAATCAT		
TaPpd-1-F		exon 1	TCCACCCGGCAGGTCGTCACCG		
TaPpd-1-inF		intron 3	TGCTTCAGTTCCTAGTTTCACTTG		
TaPpd-1-inR		exon 5	TCTTTTGGTTTCTGGCATTTTT		
TaPpd-1-ex6R1		exon 6	CATGTCGTTGTTGTTGCTGCT		
TaPpd-1-seqR1		exon 7	CCTTCTTCCCGAAGTTCC		
Primers for identifying *Ppd-1* alleles					
TaPpd-A1prodelF	*Ppd-A1*	5' UTR	CGTACTCCCTCCGTTTCTTT	57°C	338 bp (*Ppd-A1a*)
TaPpd-A1prodelR3		5' UTR	AATTTACGGGGACCAAATACC		299 bp (*Ppd-A1b*)
TaPpd-A1prodelR2		5' UTR	GTTGGGGTCGTTTGGTGGTG		
TaPpd-B1proinF1	*Ppd-B1*	5' UTR	CAGCTCCTCCGTTTGCTTCC	70°C, 60°C	620 bp (*Ppd-B1a*)
TaPpd-B1proinR1		5' UTR	CAGAGGAGTAGTCCGCGTGT		312 bp (*Ppd-B1b*)
TaPpd-D1-F1	*Ppd-D1*	5' UTR	ACGCCTCCCACTACACTG	54°C	315 bp (*Ppd-D1a*)
TaPpd-D1-R1		5' UTR	TGTTGGTTCAAACAGAGAGC		415 bp (*Ppd-D1b*)
TaPpd-D1-R2		exon 1	CACTGGTGGTAGCTGAGATT		

[a] Initial and final etmperature is shown for touchdown PCR.

Results

Evaluation of heading date in the field

Heading date of the two parental lines "Winter-Abukumawase" and "Chihokukomugi" was 7.5 April and 29.1 April, respectively, and the difference was 21.6 days. The frequency distribution of the DH population is shown in Fig. 5. It ranged from 8.2 April to 2.8 May among 80 DH lines, and its range was nearly equivalent to that between the parental lines. In addition, it showed continuous distribution, indicating the segregation of several genes.

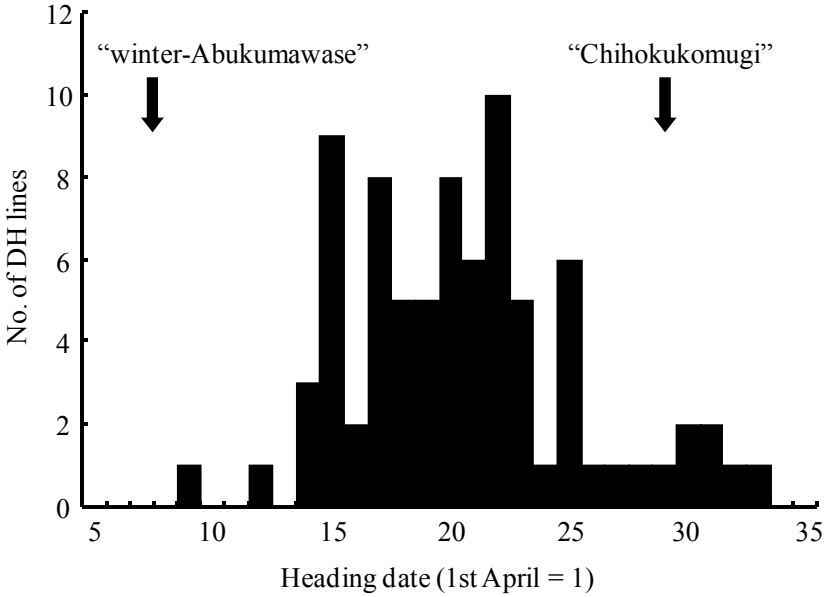

Fig. 5. Segregation of heading date in 80 DH lines. Average heading date of two parental lines was shown by arrows.

Estimation of *Ppd-1* genotype by bulk segregant analysis of linked SSR markers

For the analysis of SSR markers considered to locate near *Ppd-1* homoeologs, nine DH lines that headed earlier than 14.5 April were selected as early group, while a late group consisted of nine DH lines that headed later than 26.4 April. Assuming that segregation of heading date was caused by allelic variation of *Ppd-1* homoeologs, the late group lines are expected to be homozygous recessive at three *Ppd-1* loci. Therefore,

if the segregation of SSR markers deviated significantly from 1:1 ratio especially in late group lines, the segregation of *Ppd-1* genes could be expected.

As shown in Table 4, all of the late group lines showed "Winter-Abukumawase" allele of *Xwmc177* (2A), while this allele was less frequent (2/9) in the early group lines. The segregation ratio in the late group lines deviated significantly from 1: 1 ratio ($\chi^2 = 9.00$, df = 1, $P \leq 0.01$). This result suggested that "Chihokukomugi" had an insensitive allele of *Ppd-A1*. Similarly, eight and nine of nine late group lines showed "Chihokukomugi" alleles of *Xgwm148* (2B) ($\chi^2 = 5.44$, df = 1, $P \leq 0.025$) and *Xgwm484* (2D) ($\chi^2 = 9.00$, df = 1, $P \leq 0.01$), suggesting that "Winter-Abukumawase" had insensitive alleles of *Ppd-B1* and *Ppd-D1*.

Table 4 Bulk segregant analysis using three SSR markers linked to the *Ppd-1* loci.

Photoperiodic response of DH lines	No. of DH lines	*Xwmc177* (2A)		*Xgwm148* (2B)		*Xgwm484* (2D)	
		P1 type[a]	P2 type[a]	P1 type[a]	P2 type[a]	P1 type[a]	P2 type[a]
Insensitive (early flowering)	9	2	7	5	4	8	1
Sensitive (late flowering)	9	9	0	1	8	0	9

[a] P1 and P2 indicate "winter-Abukumawase" and "Chihokukomugi", respectively.

Sequence polymorphism of *Ppd-1* homoeologs

By using two primers, TaPpd-A1proF3 and TaPpd-1-3'UTRR3, *Ppd-A1* sequence from 5' flanking region to 3' UTR was amplified from two parental lines, "Winter-Abukumawase" and "Chihokukomugi", and the full sequence was determined. This primer set was expected to amplify 6634 bp of *Ppd-A1* sequence in "Chinese Spring", which included 2184 bp upstream from the translation start codon and 67 bp downstream from the translation stop codon. Total length of the PCR amplicon differed among the two lines, being 5412 bp and 4327 bp, respectively, and was also different from that of "Chinese Spring". The sequence from the translation start codon to the 3' UTR was identical among the three lines, except for the insertion of 1221 bp in 5th intron of "Chinese Spring" (Beales et al. 2007), and encoded a total of 668 amino acids. In contrast, in the 5' upstream sequence, a large deletion of 1085 bp between nucleotides -1420 and -336 was detected in "Chihokukomugi" which was suggested to have an insensitive allele of *Ppd-A1a.1* (Fig. 6A). Two SNPs were also detected in the 5' upstream sequence of "Chihokukomugi", whereas 5' upstream sequence was identical

between “Winter-Abukumawase” (*Ppd-A1b.2*) and “Chinese Spring” (*Ppd-A1b.1*) except two In/Dels of 1bp.

The primers TaPpd-B1proF1 and TaPpd-B1-3'UTRR1 amplified the expected 4225 bp of *Ppd-B1* sequence in “Chinese Spring”, which included 1056 bp of 5’ upstream sequence and 119 bp of 3’ UTR. The total length of the PCR amplicon was the same in “Chihokukomugi”, but was 4533 bp in “Winter-Abukumawase”. The sequence from the translation start codon to the 3’ UTR was identical among the three lines except for a non-synonymous SNP in the 3rd exon of “Chinese Spring” (Beales et al. 2007) and a synonymous SNP in 7th exon of “Chihokukomugi”, and encoded a total of 664 amino acids (Fig. 6B). However, in the 5’ upstream sequence, an insertion of 308 bp between nucleotides -734 and -733 was detected in “Winter-Abukumawase” which was considered to have an insensitive allele of *Ppd-B1a.1*. A SNP was also detected in 5’ upstream sequence of “Winter-Abukumawase”, whereas 5’ upstream sequence was identical between “Chihokukomugi” (*Ppd-B1b.1*) and “Chinese Spring” (*Ppd-B1a.2*).

By using two primers, TaPpd-D1-proF3 and TaPpd-1-3’UTRR4, we expected to amplify 6753 bp of *Ppd-D1* sequence in “Chinese Spring”, which included 3566 bp of 5’ upstream sequence and 45 bp of 3’ UTR. Total length of the PCR amplicon differed among the two parental lines, being 4660 bp and 6748 bp in “Winter-Abukumawase” and “Chihokukomugi”, respectively, and was also different from that of “Chinese Spring”. The sequence from the translation start codon to the 3’ UTR was identical among the three lines except for a deletion of 5 bp in 7th exon of “Chihokukomugi” and a non-synonymous SNP in 7th exon of “Winter-Abukumawase” and “Chihokukomugi”, and encoded a total of 660 amino acids in “Chinese Spring” and “Winter-Abukumawase” (Fig. 6C). The deletion of 5 bp, which was also known in “Norstar” (Beales et al. 2007), caused a frame-shift and leaded to a premature stop codon after 48 amino acids (amino acid 470). This resulted in truncation of 191 C-terminal amino acids, which included CCT domain. In the 5’ upstream sequence, a large deletion of 2089 bp between nucleotides -2146 and -58 was detected in “Winter-Abukumawase” which was suggested to have an insensitive allele of *Ppd-D1a.1*. This deletion was identical to that of “Ciano 67” (Beales et al. 2007). A SNP and a SSR polymorphism were also detected in 5’ upstream sequence of “Winter-Abukumawase”, whereas 5’ upstream sequence was identical between “Chihokukomugi” and “Chinese Spring”. A 16 bp deletion in exon 8, reported by Beales et al. (2007), was also confirmed in both cultivars.

Marker development and genotyping of DH lines

Three primers, TaPpd-A1prodelF1, TaPpd-A1prodelR3 and TaPpd-A1prodelR2 were used to detect the deletion of 1085 bp in the 5' upstream sequence of *Ppd-A1* (Table 5). TaPpd-A1prodelF1 and TaPpd-A1prodelR2 were designed outside of the deletion and TaPpd-A1prodelR3 inside of the deletion. By multiplex PCR, a fragment of 338 bp was amplified in "Chihokukomugi", carrier of *Ppd-A1a.1*, with primers TaPpd-A1prodelF1 and TaPpd-A1prodelR2 (Fig. 7A). A fragment of 299 bp was amplified in "Winter-Abukumawase", carrier of *Ppd-A1b.2*, with primers TaPpd-A1prodelF1 and TaPpd-A1prodelR3. Of the 80 DH lines analyzed, 44 lines and 36 lines proved to have *Ppd-A1a.1* and *Ppd-A1b.2*, respectively, and the segregation ratio fitted to 1:1 ($\chi^2 = 0.80$, df = 1, NS).

Two primers, TaPpd-B1proinF1 and TaPpd-B1proinR1, were used to detect the insertion of 308 bp in the 5' upstream sequence of *Ppd-B1* (Table 5). As shown in Fig. 7B, a fragment of 620 bp was amplified in "Winter-Abukumawase", carrier of *Ppd-B1a.1*, while a fragment of 312 bp was amplified in "Chihokukomugi", carrier of *Ppd-B1b.1*. Of the 80 DH lines analyzed, 36 lines and 44 lines proved to have *Ppd-B1a.1* and *Ppd-B1b.1*, respectively, and the segregation ratio fitted to 1:1 ($\chi^2 = 0.80$, df = 1, NS).

To detect the deletion of 2089 bp in 5' upstream sequence of *Ppd-D1*, three primers, Ppd-D1-F1, Ppd-D1-R1 and Ppd-D1-R2, designed by Beales et al. (2007) were used. As shown in Fig. 7C, a fragment of 315 bp was amplified in "Winter-Abukumawase", carrier of *Ppd-D1a.1*, while a fragment of 415 bp was amplified in "Chihokukomugi", carrier of *Ppd-D1b.2*. Of the 80 DH lines analyzed, 45 lines and 35 lines proved to have *Ppd-D1a.1* and *Ppd-D1b.2*, respectively, and the segregation ratio fitted to 1:1 ($\chi^2 = 1.25$, df = 1, NS).

A total of 80 DH lines were successfully classified into eight genotypes, by structural analysis of *Ppd-1* homoeologs (Table 5). The frequency of eight genotypes did not differ significantly from the expected ratio ($\chi^2 = 6.60$, df = 7, NS), though the *Ppd-A1b.2* / *Ppd-B1a.1* / *Ppd-D1b.2* genotype was rather less frequent.

Relationship between *Ppd-1* genotype and heading date

The average heading date differed significantly among genotypes, and ranged from 17.04 for the *Ppd-A1a.1* / *Ppd-B1b.1* / *Ppd-D1a.1* genotype to 29.21 for the *Ppd-A1b.2* / *Ppd-B1b.1* / *Ppd-D1b.2* genotype (Table 5). It was the earliest, from 17.04 to 17.95, in four genotypes carrying two or three *Ppd-1* genes causing insensitivity to photoperiod, while the latest (*Ppd-A1b.2* / *Ppd-B1b.1* / *Ppd-D1b.2* genotype) carried no insensitive genes. Heading was accelerated by 7-9 days with each of the three insensitive genes, since the heading date of three genotypes carrying one of the three insensitive genes ranged from 20.31 to 22.13. Among them, *Ppd-A1a.1* / *Ppd-B1b.1* / *Ppd-D1b.2* genotype seemed to be late heading compared with *Ppd-A1b.2* / *Ppd-B1a.1* / *Ppd-D1b.2* and *Ppd-A1b.2* / *Ppd-B1b.1* / *Ppd-D1a.1* genotypes, though the difference was insignificant.

Table 5 Number of DH lines classified into eight genotypes by PCR assays and average heading date of each genotype.

Genotype[a]			No. of	Heading date[b]	
Ppd-A1	*Ppd-B1*	*Ppd-D1*	DH lines	Average	SE
a	*a*	*a*	12	17.28 a	0.700
a	*a*	*b*	12	17.95 ab	0.429
a	*b*	*a*	11	17.04 a	0.472
b	*a*	*a*	8	17.53 ab	0.715
a	*b*	*b*	9	22.13 c	0.443
b	*a*	*b*	4	20.59 bc	0.499
b	*b*	*a*	14	20.31 bc	0.519
b	*b*	*b*	10	29.21 d	0.463

[a] 'a' and 'b' indicate photoperiod-insensitive and -sensitive allele of *Ppd-1* genes, respectively.

[b] 1st April = 1. Values with the different letter indicate significant difference ($P<0.01$) by Tukey test.

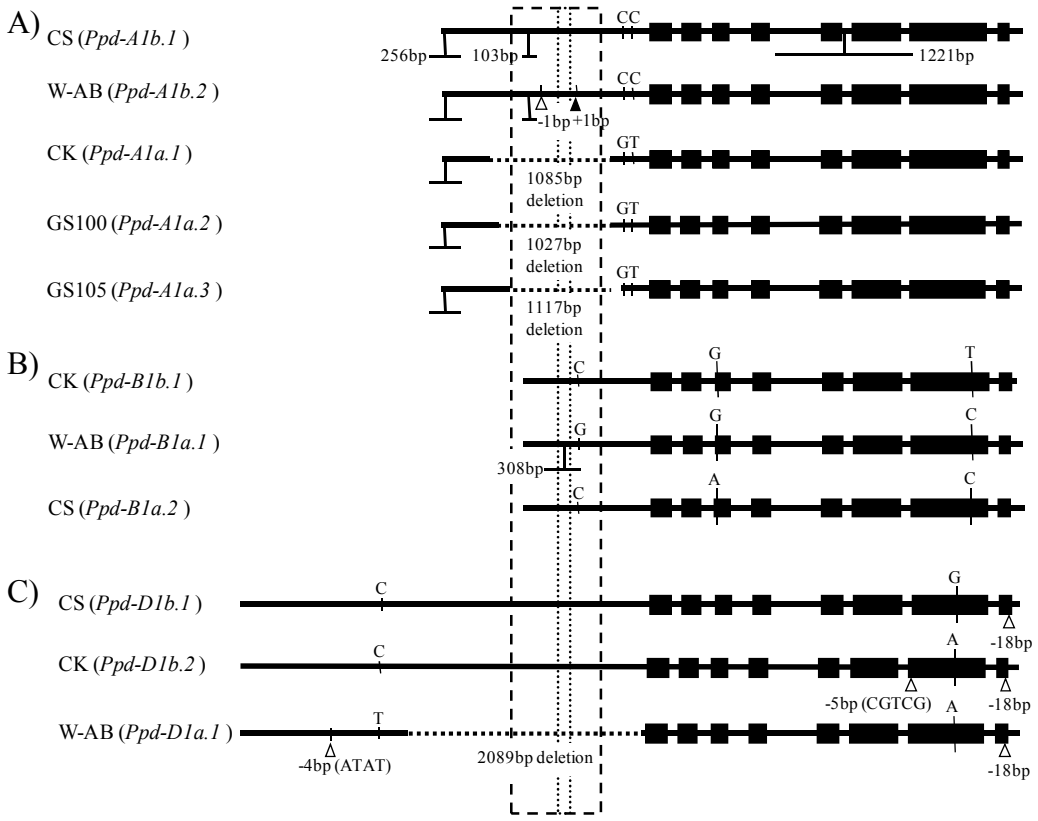

Fig. 6. Structural difference of *Ppd-1* homoeologs among three cultivars Chinese Spring (CS), Winter-Abukumawase (W-AB), and Chihokukomugi (CK). Photoperiod-insensitive alleles (indicated by the "*a*" suffix) except *Ppd-B1a.2* have in/dels in 5' upstream region, which do not exist in photoperiod-sensitive alleles (indicated by the "*b*" suffix). A) *Ppd-A1a.1* of CK has a 1085bp deletion at the position -1420 to -336 in CS. B) *Ppd-B1a.1* of W-AB has a 308bp insertion at the position between -734bp and -733bp in CS. C) *Ppd-D1a.1* of W-AB has a 2089bp deletion located at the position -2146 to -58bp in CS. A 5bp deletion in exon 7 of CK *Ppd-D1b.2* is the same as that of Norstar (Beales et al. 2007). A 18bp deletion in exon 8 of *Ppd-D1* gene is also reported by Beales et al. (2007).

Filled boxes and horizontal lines between them indicate exons and introns respectively. Open and filled triangles indicate the position of small deletions and insertions. Dotted line indicates large deletion within the 5' upstream region. Thin vertical line indicates the position of single nucleotide polymorphism (SNP). Horizontal line on the bottom end of thick vertical line indicates transposon. Ca. 900bp region commonly deleted in photoperiod-insensitive alleles of *Ppd-A1* and *Ppd-D1* and specially conserved ca. 100bp region (Wilhelm et al. 2009) are indicated by boxes of broken line and dotted line, respectively.

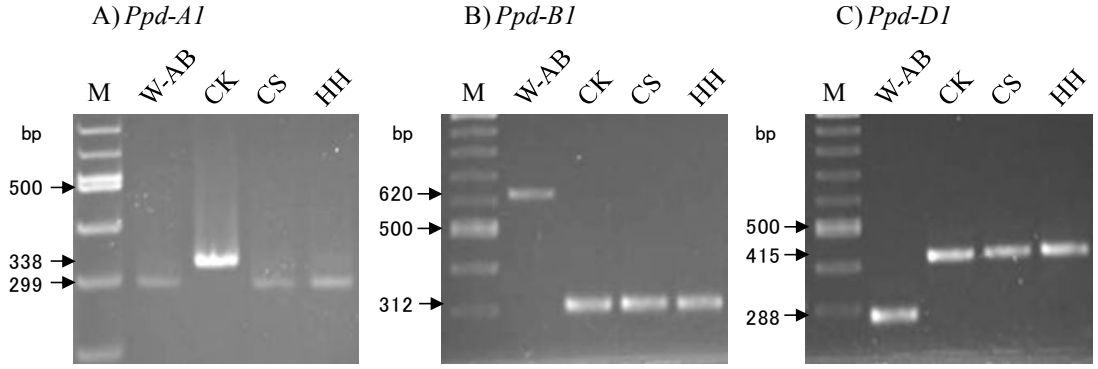

Fig. 7. PCR assays to identify *Ppd-1* genotype of four cultivars "Winter-Abukumawase" (W-AB), "Chihokukomugi" (CK), "Chinese Spring" (CS) and "Haruhikari (HH)". A) *Ppd-A1a.1* allele of CK (338bp) is clearly distinguished by a multiplex PCR to amplify 5' upstream region of *Ppd-A1*. B) *Ppd-B1a.1* allele of W-AB (620bp) is clearly distinguished by amplifying 5' upstream region of *Ppd-B1*. C) *Ppd-D1a.1* allele of W-AB (415bp) is clearly distinguished by a multiplex PCR to amplify 5' upstream region of *Ppd-D1*.

Discussion

Ppd-1 homoeologs controlling photoperiod sensitivity are located on homoeologous group 2 chromosomes in wheat and barley (Snape et al. 2001; Laurie et al. 1995). Dominant alleles confer photoperiod insensitivity, while recessive alleles confer photoperiod sensitivity. In wheat, it is known that a photoperiod-insensitive allele of *Ppd-B1* is carried by "Chinese Spring", "Spica", and "Timstein" (Hoogendoorn 1985; Scarth and Law 1983; Scarth and Law 1984), whereas that of *Ppd-D1* is carried by "Akakomugi", "Bezostaya 1", "Mara", "Recital", "Saitama 27", "Sonora 64", and "Norin 61" (Welsh et al. 1973; Worland et al. 1988; Tanio et al. 2005; Beales et al. 2007). As to *Ppd-A1*, on the contrary, the insensitive allele has not been found until Wilhelm et al. (2009) detected two types of insensitive alleles of *Ppd-A1* in *T. durum*. The present study is the first report of an insensitive allele of *Ppd-A1* in common wheat. "Chihokukomugi" is a Japanese winter type cultivar which used to be grown widely in Hokkaido area, with the latitude over 41°N, during 1988 - 1996.

The comparison of genomic sequence of *Ppd-A1* revealed that it was identical from the translation start codon to the 3'UTR among *Ppd-A1a.1* ("Chihokukomugi") and *Ppd-A1b.2* ("Winter-Abukumawase") alleles. Both sequences lack an insertion of transposon (ca. 1.2 kb) in intron 6 reported in "Chinese Spring" (*Ppd-A1b.1*). Sequence polymorphism was detected in the 5' upstream region, where a large deletion of 1085 bp between nucleotides -1420 and -336 was detected in *Ppd-A1a.1* ("Chihokukomugi") (Fig.

6A). The deletion was flanked by a short repeated sequence of 4bp, GATT, suggesting that a novel allele of *Ppd-A1* was created by error prone nonhomologous end-joining of double-stranded breasks as reported for *Vrn-1* homoeologs of wheat (Cockram et al. 2007). In *T. durum*, two types of deletion differing in size are known also in the 5' upstream region of *Ppd-A1*. Although gene (allele) symbol has not been given to durum alleles, the insensitive alleles with a deletion of 1027 bp ("GS-100") and 1117 bp ("GS-105") should be designated as *Ppd-A1a.2* and *Ppd-A1a.3*, respectively. As shown in Fig. 6A, the three large deletions found in insensitive alleles of *Ppd-A1* shared the common region between nucleotide -1193 and -336. They also shared this common region (ca. 900 bp) with the deletion of *Ppd-D1a.1* (Fig. 6C, Beales et al. 2007). Wilhelm et al. (2009) hypothesized that there is regulatory element(s) in this region which is required for precise diurnal expression pattern and photoperiodic response under short photoperiods. They further suggested that approximately 100 bp region, 120 bp upstream of transcription start site, within the common 900 bp region is especially important, because it is well conserved among barley, rice and *Brachypodium*. *Ppd-A1a.1* in this study lacks the especially important 100 bp region (actually 84bp) and most of the common 900 bp region except for 35 bp in 3' end.

Beales et al. (2007) found several polymorphisms within *Ppd-B1* genes of hexaploid wheat, including five SNPs and a retrotransposon insertion. However, none corresponds with the photoperiodic response, implying that the critical mutation causing allelic difference of *Ppd-B1* gene has not been found. The present study is the first report of a likely causal mutation in the *Ppd-B1* gene as a 308 bp insertion was identified in the 5' upstream region of *Ppd-B1a.1* of "Winter-Abukumawase" (Fig. 6B), which proved to carry *Ppd-B1a.1* as well as *Ppd-D1a.1*. The 308 bp insertion sequence has a target site duplication (TTA) and terminal inverted repeats of 21 bp at both ends, and was considered as a miniature inverted repeat transposable element (MITE). The especially important 100 bp region (actually 95bp) was interrupted by this insertion, suggesting strongly that this might be a causal mutation for photoperiod-insensitivity. For further understanding of the molecular mechanism of photoperiod-insensitivity, expression analysis of *Ppd-1* homoeologs and other related genes and precise evaluation of photoperiodic response should be conducted using the DH lines with different *Ppd-1* genotypes.

Tanio and Kato (2007) analyzed the effect of *Ppd-1* genes on photoperiodic response using near isogenic lines of "Haruhikari", a Japanese photoperiod-sensitive cultivar. They reported that the insensitive allele of *Ppd-B1* introduced from "Fukuwasekomugi" has a stronger effect on reducing photoperiod sensitivity than *Ppd-D1a* of

"Fukuwasekomugi" and "Saitama 27". "Fukuwasekomugi" and "Winter-Abukumawase" are sister cultivars derived from a common early heading line "Chugoku 114" and they indicated that the breeding of early maturing cultivar has been accomplished by combining two insensitive alleles, *Ppd-B1a* and *Ppd-D1a*. However, their result was contradicting with those of Scarth and Law (1984) and Gonzalez et al. (2005), who reported that *Ppd-D1a* of "Ciano 67" had a stronger effect than *Ppd-B1a* of "Chinese Spring". In addition, according to Scarth and Law (1984), the insensitive allele of *Ppd-B1* carried by "Chinese Spring" has a weaker effect than that of "Timstein". Although *Ppd-B1* of "Timstein" has not been sequenced, the stronger allele *Ppd-B1a.1* of "Winter-Abukumawase" has the insertion in 5' upstream region (Fig. 6B), while the weaker allele *Ppd-B1a.2* of "Chinese Spring" does not (Beales et al. 2007). It was therefore indicated that *Ppd-B1* locus has multiple alleles and the effect of *Ppd-B1* alleles on photoperiod-insensitivity is strongly affected by the insertion in 5' upstream region.

According to Tanio and Kato (2007), photoperiodic response was the smallest in *Ppd-B1a / Ppd-D1a* NIL, followed by *Ppd-B1a / Ppd-D1b* NIL, *Ppd-B1b / Ppd-D1a* NIL, and *Ppd-B1b / Ppd-D1b* NIL. This result indicates the stronger effect of *Ppd-B1a.1* and the interaction between *Ppd-B1a.1* and *Ppd-D1a.1*. As shown in Table 5, in the present study, DH lines carrying two or three insensitive genes proved to be the earliest in heading, suggesting an interaction among *Ppd-1* genes. As to the effect of each insensitive allele of *Ppd-1* genes, *Ppd-B1a.1* and *Ppd-D1a.1* confer the same magnitude of photoperiod insensitivity, which seems stronger than that of *Ppd-A1a.1*. Although this result is contradicting with Tanio and Kato (2007), the donor cultivars of *Ppd-D1a*, "Saitama 27" in Tanio and Kato (2007) and "Winter-Abukumawase" in the present study, carried the same *Ppd-D1a.1* allele without a 5 bp deletion in exon 7 and *Ppd-B1a.1* derived from their common parent "Chugoku 114" (Seki et al. in preparation). Therefore, the discrepancy might be ascribable to the difference in the genetic background, including earliness *per se* QTL linked to *Ppd-1* loci (Shindo et al. 2003).

Ppd-A1a.1 of "Chihokukomugi" and *Ppd-B1a.1* of "Winter-Abukumawase" are novel photoperiod insensitive alleles found in this study. They seem unique to the Japanese wheat cultivars, though further study is required. Photoperiodic response is the major determinant of heading time (Kato and Yamashita 1991) and insensitive alleles of *Ppd-1* genes show significant interactions (Table 5). Therefore, these new alleles for photoperiod insensitivity can be utilized as novel genetic resources for the breeding of early heading cultivars, like *Ppd-D1a* introduced from Japanese cultivars "Akakomugi" and "Saitama 27" to European wheats (Worland et al. 1996). The PCR-based markers

established in this study can be applicable to the marker assisted selection (MAS) of *Ppd-1* genes, since a diagnostic PCR band identifies the sensitive or insensitive allele of each *Ppd-1* gene.

Recent studies on circadian clock genes and clock-controlled genes in Arabidopsis revealed that they share some kind of cis-elements or motifs. We searched for such sequences in the common region of tetraploid *Ppd-A1a*, hexaploid *Ppd-A1a* and *Ppd-D1a* deletions, and found five kinds of cis-elements or motifs: a G-box core that intermediates light-response, a G-box related Hex element that exists in the promoter of circadian-regulated genes expressing around dawn (Schindler et al. 1992), a consensus sequence that is often found with Hex element and matches exactly to a part of G-box core (Michael and McClung 2003), two binding sites for CHE (CCA1 HIKING EXPEDITION; a transcription repressor and acts redundant with LHY and interacts with TOC1 (Pruneda-Paz et al. 2009)), and a refined morning element that was found to be associated with morning specific expression by the analysis of large-scale microarray set (Michael et al. 2008).

As to *Ppd-B1*, we sequenced most of the region corresponding to the common region of tetraploid *Ppd-A1a*, hexaploid *Ppd-A1a* and *Ppd-D1a* deletions, although the 5' end of the region was not covered (Fig. 6). The unsequenced region was estimated to be 133bp based on CS *Ppd-A1b.1* sequence and there were no motifs in this region of *Ppd-A1* and *Ppd-D1*. The result showed that *Ppd-B1a* and *Ppd-B1b* lack G-box core and consensus sequence because of the SNP. Instead, they have a SORLIP1 site (a Sequence Overrepresented in Light-Induced Promoters; Hudson and Quail 2003) that is related to morning element. Similar to *Ppd-A1* and *Ppd-D1*, they have a refined morning element and two CHE binding sites, although the CHE binding sites are separated by the 308bp insertion in *Ppd-B1a.1* that is located 29bp upstream of 2nd CHE binding site. In addition, there are two 5A motifs (Spensley et al. 2009) that is introduced by the 308bp insertion. This motif was shown to be associated with circadian gene expression.

According to Wilhelm et al. (2009), there is an approximately 100bp sequence within the common deletion region which is well conserved among some Poaceae species like wheat, barley, *Brachypodium*, and rice. They speculated that there might be some motifs regulating transcription in this region, where we found a CHE binding site and a Hex element. As mentioned above, *Ppd-A1a* and *Ppd-D1a* lack both motifs by the large deletion, while *Ppd-A1b* and *Ppd-D1b* have intact motifs. Furthermore, *Ppd-B1a.1* has the 308bp insertion that separates the two CHE binding sites, while, in *Ppd-B1b*, CHE binding sites were close as in *Ppd-A1b* and *Ppd-D1b*. This suggested that either of them might play important roles in regulating diurnal expression of *Ppd-1*, although it is not

sure that these motifs found in Arabidopsis function in the same way in wheat.

Abstract

For genetic analysis of *Ppd-1* homoeologs controlling photoperiodic response of wheat, bulk segregant analysis was conducted using a DH population derived from a cross of two Japanese wheat genotypes, "Winter-Abukumawase" and "Chihokukomugi". Based on the segregation of SSR markers linked to the *Ppd-1* homoeologs, "Winter-Abukumawase" carried two insensitive alleles (*Ppd-B1a* and *Ppd-D1a*) while "Chihokukomugi" carried a single insensitive allele (*Ppd-A1a*) which was first found in common wheat. Genomic sequence of *Ppd-1* homoeologs, including 5' upstream region, was determined and compared among two genotypes. *Ppd-D1a* of "Winter-Abukumawase" had a deletion of 2089 bp which was already reported in "Ciano 67". Critical sequence polymorphism causing photoperiod insensitivity was not detected from the translation start codon to 3' UTR of *Ppd-A1* and *Ppd-B1*. However, novel mutations were found in the 5' upstream region. *Ppd-A1a* of "Chihokukomugi" had a deletion of 1085 bp and *Ppd-B1a* of "Winter-Abukumawase" had an insertion of 308 bp. A total of 80 DH lines were classified into eight genotypes by PCR based genotyping using specific primer sets to detect the In/Del in 5' upstream region of three *Ppd-1* genes. Heading date of DH lines differed significantly among eight genotypes, showing that heading was accelerated by 7-9 days, compared with photoperiod sensitive genotype, with each of the three insensitive alleles. Interaction between three genes was also significant.

CHAPTER Ⅳ. General discussion

In Japan, poor grain quality of wheat will be the problem when intermediate- and late-heading varieties at maturing stage encounter intermittent rain during rainy season. To avoid this problem, early-heading varieties have been developed through the history of wheat breeding in Japan. However, such early-heading varieties often suffer from frost injury during winter and sterility by cold temperature around flowering time, resulting in low grain yield. The effective way to avoid these problems and improve both of yield and quality is to make heading time suitable to local climatic conditions where the varieties will be cultivated. To achieve this, heading time-related genes need to be identified. To date, vernalization requirement genes *Vrn-1*, *Vrn-2*, and *Vrn-3* have been cloned and sequence variations that explain allelic difference have been discovered (Yan et al.2003, 2004b, 2006). Photoperiodic response gene *Ppd-1* has also been cloned and sequence variation that characterizes allelic difference has been discovered in *Ppd-D1* (Beales et al. 2007). However, among four vernalization requirement genes, *Vrn-4* is the only gene which has not been cloned. As for *Ppd-1*, Sequence variation that explains the allelic difference has not been identified in *Ppd-A1* and *Ppd-B1*. This study focused on the molecular genetic analysis on these heading time genes as described in the followings.

In chapter 2, effect of *Vrn-D4* was analyzed by comparing vernalization requirement among Triple Dirk NILs with different genotype for this trait. The result clearly indicated that the NIL which carries the spring allele *Vrn-D4* (TDF-J) did not require cold temperature for transition from vegetative growth to reproductive growth, while the winter type NIL which does not carry any spring allele (TDC) required it (Fig. 2). This result confirmed that *Vrn-D4* surely controls vernalization requirement and functions within the vernalization pathway. The result also showed that the NIL carrying *Vrn-D4* (TDF-J) still had residual vernalization response to some extent. This is similar to the NILs carrying spring alleles *Vrn-B1* (TDB) and *Vrn-D1* (TDE) (Berry et al. 1980; Pugsley 1972). However, vernalization responses were different among these three NILs, especially when duration of vernalization treatment is shorter and vernalization requirement is not satisfied. When duration of the treatment is 5 to 10 days, days to flag leaf unfolding was decreased by 5 to 10 days in the NILs carrying *Vrn-B1* (TDB) and *Vrn-D1* (TDE), while it was not in the NIL carrying *Vrn-D4* (TDF-J).

Effect of *Vrn-D4* on expression of other genes involved in vernalization requirement was also analyzed. TDF-J carrying *Vrn-D4* expressed *Vrn-1* and *Vrn-3* at higher level and *Vrn-2* at lower level than CS($5D_{5402}$) when they were sown at the same time (Table 2). This suggested that *Vrn-D4* might function upstream of or within the feedback regulatory loop which consists of *Vrn-1*, *Vrn-2*, and *Vrn-3*.

So far, preliminary genetic study has disclosed that *Vrn-D4* is located on chromosome

5D (Kato et al. 1993) and it is closely linked by the SSR marker *Xgdm3* on the centromeric region of the same chromosome (Kato et al. 2003). In this study, fine mapping of *Vrn-D4* made it clear that *Vrn-D4* cosegregated with the SSR marker *Xcfd67* and the EST-SNP marker *XBG313707*. These cosegregating markers will be effective for marker assisted selection in the practical breeding program to introduce *Vrn-D4*. However, there are generally less recombination events in the centromeric region compared with other regions, which make positional cloning difficult. Then, comparative mapping analysis with rice and marker development based on the analysis will be required for *Vrn-D4* cloning.

In chapter 3, molecular cloning and sequence analysis were conducted on photoperiod-insensitive alleles of *Ppd-1* homoeologs. Initially, bulked segregant analysis was conducted to make it clear whether the DH population from a cross of winter-type Abukumawase and Chihokukomugi segregates *Ppd-1* homoeologs by using SSR markers linked to them. The result revealed that the DH segregated all of three homoeologs, and that winter-type Abukumawase carries two photoperiod-insensitive alleles *Ppd-B1a* and *Ppd-D1a* while Chihokukomugi carries a photoperiod-insensitive allele *Ppd-A1a* (Table 4). Recently, it was reported that *Ppd-B1a* contributed to develop extremely early varieties by introducing it into intermediate varieties carrying *Ppd-D1a* in Japan, and the *Ppd-B1a* was derived from a Japanese landrace Shiroboro 21 (Tanio et al. 2005, Kato et al. 2005, Seki et al. 2011). On the other hand, as there had not been any reports on *Ppd-A1a* before, this study is the world-first report on *Ppd-A1a*.

Phenotypic effect of photoperiod-insensitive alleles was analyzed by comparing heading time under field condition among the DH lines. Each allele promoted heading time by more than 1 week, but extent of effect was different among alleles (Table 5). Their promoting effect was estimated to be *Ppd-B1a* = *Ppd-D1a* > *Ppd-A1a*. In addition, there were interactions among alleles, supporting the conclusion that extremely early varieties have been developed by introducing *Ppd-B1a* into intermediate varieties carrying *Ppd-D1a* (Tanio et al. 2005; Kato et al., 2005). Because the effect of *Ppd-A1a* was smaller than other photoperiod-insensitive alleles, *Ppd-A1a* is expected to be an unique gene resource for fine tuning of heading time.

Sequencing analysis showed that *Ppd-A1a* and *Ppd-B1a* coding sequences are identical with *Ppd-A1b* and *Ppd-B1b* respectively. On the contrary, polymorphisms were found in their upstream region: a 308 bp insertion in *Ppd-B1a* (the allele from winter type Abukumawase) and a 1085 bp deletion in *Ppd-A1a* (the allele from Chihokukomugi) (Fig. 6). Similar to *Ppd-A1* and *Ppd-B1*, *Ppd-D1a* has the identical coding sequence with *Ppd-D1b* and a 2089bp deletion in its upstream region which

Ppd-D1b does not have, thereby its circadian expression is disrupted (Beales et al. 2007). The insertion in *Ppd-B1a* and deletion in *Ppd-A1a* lie on the region overlapping with the deletion in *Ppd-D1a*. This overlapping region is well conserved among *Poaceae* species, tetraploid wheat, barley, rice, and *Brachypodium*, suggesting disruption of circadian expression in *Ppd-B1a* and *Ppd-A1a* by the insertion and the deletion respectively.

In Arabidopsis, function of several photoperiodic response genes has been elucidated by molecular genetic studies. Based on their results, some models on genetic pathways for photoperiodic response have been proposed. Basically, they consist of genes for photoreceptors, circadian clock, integrator and transmitter of signals from photoreceptors and circadian clock, and flowering promoter. There are five *PRR* genes (*TOC1, APRR3, APRR5, APRR7, APRR9*) in Arabidopsis. Among them, *TOC1* , together with CHE, plays central role in circadian clock (Strayer et al. 2000, Mizuno and Nakamichi 2005, Zeilinger et al. 2006, Pruneda-Paz et al. 2009). *Ppd-1* is also the *PRR* gene and shows circadian expression, suggesting its association with circadian clock. By motif analysis, two CHE binding sites were found in upstream region of photoperiod-sensitive *Ppd-1* homoeoalleles. On the contrary, both of them are absent in *Ppd-A1a* and *Ppd-D1a* by large deletions, and an insertion exists between them in *Ppd-B1a*. This fact suggested that two CHE binding sites may play an important role in circadian expression of *Ppd-1* as it does in *TOC1*. To reveal the function of *Ppd-1* in the genetic pathway for photoperiodic response, detailed analysis on expression pattern of *Ppd-1* homoeologs and comprehensive expression analysis on orthologs of Arabidopsis photoperiodic response genes will be required.

ACKNOWLEDGMENTS

I would like to express my deep gratitude to Professor Kenji Kato and Assistant Professor Hidetaka Nishida at Laboratory of Plant Genetics and Breeding for their kind and careful guidance and supervision all through this study until completion of the Dr. thesis. I am also grateful to Dr. Yukari Akashi, Professor Jorge Dubcovsky at University of California, Davis, and Dr. David A. Laurie at John Innes Centre for their valuable advices and detailed guidance. I would like to express my appreciation to members of Laboratory of Plant Genetics and Breeding for their advices and cooperation on this study.

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