Genes Genet. Syst. (2012) **87**, p. 161–168

Effect of *Ppd-1* on the expression of flowering-time genes in vegetative and reproductive growth stages of wheat

Satoshi Kitagawa, Sanae Shimada and Koji Murai*

Department of Bioscience, Fukui Prefectural University, 4-1-1 Matsuoka-kenjojima, Eiheiji-cho, Yoshida-gun, Fukui 910-1195, Japan

(Received 16 January 2012, accepted 23 April 2012)

The photoperiod sensitivity gene *Ppd-1* influences the timing of flowering in temperate cereals such as wheat and barley. The effect of *Ppd-1* on the expression of flowering-time genes was assessed by examining the expression levels of the vernalization genes *VRN1* and *VRN3/WFT* and of two *CONSTANS*-like genes, *WCO1* and *TaHd1,* during vegetative and reproductive growth stages. Two near-isogenic lines (NILs) were used: the first carried a photoperiod-insensitive allele of *Ppd-1* (*Ppd-1a*-NIL), the other, a photoperiod-sensitive allele (*Ppd-1b*-NIL). We found that the expression pattern of *VRN1* was similar in *Ppd-1a*-NIL and *Ppd-1b*-NIL plants, suggesting that *VRN1* is not regulated by *Ppd-1*. Under long day conditions, *VRN3/WFT* showed similar expression patterns in *Ppd-1a*-NIL and *Ppd-1b*-NIL plants. However, expression differed greatly under short day conditions: *VRN3/WFT* expression was detected in *Ppd-1a*-NIL plants at the 5-leaf stage when they transited from vegetative to reproductive growth; very low expression was present in *Ppd-1b*-NIL throughout all growth stages. Thus, the *Ppd-1b* allele acts to down-regulate *VRN3/WFT* under short day conditions. *WCO1* showed high levels of expression at the vegetative stage, which decreased during the phase transition and reproductive growth stages in both *Ppd-1a*-NIL and *Ppd-1b*-NIL plants under short day conditions. By contrast to *WCO1*, *TaHd1* was up-regulated during the reproductive stage. The level of *TaHd1* expression was much higher in *Ppd-1a*-NIL than the *Ppd-1b*-NIL plants, suggesting that the *Ppd-1b* allele down-regulates *TaHd1* under short day conditions. The present study indicates that down-regulation of *VRN3/WFT* together with *TaHd1* is the cause of late flowering in the *Ppd-1b*-NIL plants under short day conditions.

Key words: flowering, photoperiod sensitivity, *Ppd-1*, *VRN1*, wheat

INTRODUCTION

The transition from vegetative to reproductive growth (flowering) is associated with heading time, one of the most important traits in cereal crops. In bread wheat (*Triticum aestivum*, 2n=6x=42, genome constitution AABBDD), heading time is genetically determined by three characteristic components, i.e., vernalization requirement, photoperiod sensitivity and narrow-sense earliness (earliness per se), which compose the autonomous promoting pathway (Worland and Snape, 2001). Of these characteristics, photoperiod sensitivity is the most important for determining heading time in autumn sown temperate cereals.

Photoperiod sensitivity is determined by the major genes *Ppd-A1*, *Ppd-B1* and *Ppd-D1* located on chromosomes 2A, 2B and 2D, respectively (Laurie, 1997). A barley (*Hordeum vulgare*, 2n=2x=14, HH) ortholog of the *Ppd-1* genes, *Ppd-H1*, was identified as a pseudo-response regulator (*PRR*) gene with a CCT domain that showed similarity to *Arabidopsis PRR7* (Turner et al., 2005). In *Arabidopsis*, the *PRR* family consists of five members (*PRR9*, *PRR7*, *PRR5*, *PRR3* and *PRR1/TOC1*) that are involved in circadian clock function together with *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*)/*LATE ELONGATED HYPOCOTYL* (*LHY*) and *GIGANTEA* (*GI*) (Nakamichi, 2011). Outputs from the circadian clock control the expression of *CONSTANS* (*CO*), a key gene of the photoperiod pathway, and up-regulate the mobile florigen gene *FLOWERING LOCUS T* (*FT*) (Imaizumi and Kay, 2006). The high sequence similarity of *Ppd-H1*

Edited by Masao Tasaka
* Corresponding author. E-mail: murai@fpu.ac.jp

with *PRR7* suggests that it is likely to be a circadian clock-associated gene. Comparative mapping indicated that wheat *Ppd-A1*, *Ppd-B1* and *Ppd-D1* are orthologous to barley *Ppd-H1*, a conclusion that is supported by sequence and expression analyses (Beales et al., 2007). *Ppd-D1* is located on chromosome 2D and confers a stronger effect on heading time than other *Ppd-1* alleles located on chromosome 2 homoeologs (Law et al., 1978). A semi-dominant photoperiod insensitive allele, *Ppd-1a*, was identified along with its photoperiod sensitive counterpart, *Ppd-1b*. A 2,089 bp deletion upstream of the coding region of *Ppd-D1a*, which causes mis-expression of the gene, is associated with photoperiod insensitivity in wheat (Beales et al., 2007). In the *Ppd-A1a* allele, a 1,027 bp or 1,117 bp deletion upstream of the coding region is associated with photoperiod insensitivity (Wilhelm et al., 2009). In the A and D genome, *Ppd-1a* alleles are more highly expressed than *Ppd-1b* alleles (Shaw et al., 2012). Contrary to *Ppd-A1* and *Ppd-D1*, there are no sequence differences (insertion/deletion) between *Ppd-B1a* and *Ppd-B1b* (Beales et al., 2007). Among three photoperiod sensitive alleles, *Ppd-A1b*, *Ppd-B1b*, and *Ppd-D1b*, the B genome was predominantly expressed (Shaw et al., 2012). In all genomes, *Ppd-1a* alleles confer photoperiod insensitivity on wheat plant, but the mechanism is still unknown.

Several near-isogenic lines (NILs) of the spring wheat cv. Triple Dirk (TD) have been established using a backcrossing method (Pugsley, 1968). The TD cultivar is photoperiod insensitive with *Ppd-D1a*. The photoperiod sensitive allele *Ppd-D1b* was introduced from the wheat cv. Selkirk and an NIL developed by backcrossing to TD. The NIL carrying a photoperiod sensitive allele of *Ppd-D1b* was originally called TD(A) (Pugsley, 1968). In a previous study, we compared flowering-times in a growth chamber of TD and TD(A) plants, in which they were named *Ppd*-NIL and *ppd*-NIL, respectively (Murai et al., 2003). This comparison showed that the *Ppd*-NIL is not completely photoperiod-insensitive and that short day (SD) conditions cause only a slightly delay in heading-time compared to long day (LD) conditions. The *ppd*-NIL shows early-heading in a similar fashion to the *Ppd*-NIL under LD conditions, but extremely late heading under SD conditions. In a subsequent study, we compared the diurnal expression patterns of flowering-time genes in the *Ppd*-NIL and *ppd*-NIL, in which they were named *Ppd*-TD and *ppd*-TD, respectively (Shimada et al., 2009). At the 3-leaf stage of vegetative growth, both *Ppd*-TD and *ppd*-TD plants showed a diurnal pattern of *VRN1* expression under both LD and SD conditions, with a peak of *VRN1* expression at the beginning of the light period. *VRN1*, a flowering promoter gene, encodes a MADS-box transcription factor that controls vernalization-induced flowering in temperate cereals (Yan et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Danyluk et al., 2003). In contrast to the expression pattern of *VRN1* in the two NILs, we found that diurnal expression of the wheat *FT* ortholog, *VRN3* (also named *WFT*), differed greatly under LD and SD conditions. In both *Ppd*-TD and *ppd*-TD plants, *VRN3/WFT* showed a diurnal expression pattern under LD conditions and a constant and very low level of expression under SD conditions.

Temperate cereals have two *CO*-like genes, *Wheat CO* (*WCO1*) (Shimada et al., 2009) and *Triticum aestivum HEADING DATE 1* (*TaHd1*) (Nemoto et al., 2003). *Hd1* is a rice ortholog of *CO* (Yano et al, 2000). At the 3-leaf stage, *Ppd*-TD and *ppd*-TD plants have similar diurnal expression patterns for these genes, although they do show a slight difference under SD conditions: *WCO1* and *TaHd1* mRNAs accumulate at higher levels in the dark in *Ppd*-TD compared to *ppd*-TD (Shimada et al., 2009). The observations summarized above indicate that the difference in flowering time phenotypes of *Ppd*-TD and *ppd*-TD at the 3-leaf stage under SD conditions cannot be ascribed to dissimilarities in the expression patterns of flowering-time genes (Shimada et al., 2009). In this study, we examined the expression patterns of *VRN1*, *VRN3/WFT*, and two *CO*-like genes, *WCO1* and *TaHd1* in the *Ppd*-TD and *ppd*-TD during the vegetative and reproductive growth phases. We found that *Ppd*-TD and *ppd*-TD plants had similar patterns of *VRN1* and *WCO1* expression; however, *VRN3/WFT* and *TaHd1* expression differed between the two genotypes at the phase transition and reproductive growth stages under SD conditions. The present results suggest that down-regulation of *VRN3/WFT* and *TaHd1* is associated with late flowering in *ppd*-NIL plants under SD conditions. In this study, *Ppd-TD* and *ppd-TD* are renamed *Ppd-1a-NIL* and *Ppd-1b-NIL*, respectively, to describe their alleles precisely.

MATERIALS AND METHODS

Plant materials Bread wheat (*Triticum aestivum*) cv. Triple Dirk (TD) lines that are nearly isogenic for the photoperiod-insensitive (*Ppd-1a*) or photoperiod-sensitive (*Ppd-1b*) alleles of *Ppd-1* were developed by Pugsley (1968). *Ppd-1b*-NIL is photoperiod-sensitive; short day (SD) conditions cause an extremely delayed heading time compared with long day (LD) conditions (Murai et al., 2003). By contrast, *Ppd-1a*-NIL is not completely photoperiod-insensitive, and SD conditions only cause a slight delay in heading time compared with LD conditions. Both NILs carry the vernalization-insensitive (spring habit) genes *Vrn-A1* and *Vrn-B1*.

Growth conditions Sprouted seeds, which had been placed on wet filter paper for 2 days at 20°C, were sown in small soil-filled containers spaced 2 cm apart. Tsuchitaro (Sumirin, Japan) was used as soil, which contains optimized amount of fertilizers. For the phenotypic study,

Table 1. Sequences of primers used for real-time PCR analyses

Gene	Primer name	Sequence (5'-3')	Annealing temperature (°C)	Extension time (sec.)
VRN1	TaMADS#11-545L	GGAGAGGTCACTGCAGGAGGA	65	10
	TaMADS#11-698R	GCCGCTGGATGAATGCTG		
VRN3/WFT	WFT-F4	CAGGCCGGTCGATCTATACTA	58	15
	WFT-R4	TCCTGTTCCCGAAGGTCA		
WCO1	CO1-LC	GCACCACTTGTAGGGGCAGA	63	16
	CO1-RC	TTGATCCTTGGCCGTGCTT		
TaHd1	CO2-2L	CCAGTACCTACACAGCTTCCA	63	16
	CO2-2R	GCCTGCTTCTTCTCCTTGT		
Ubiquitin	Ubi-1L	GCATGCAGATATTTGTGA	58	15
	Ubi-1R	GGAGCTTACTGGCCAC		

non-vernalized plants were grown in a growth chamber, LH-350S (NK system, Japan), under continuous light (24 h light), long day (16 h light/8 h dark) or short day (10 h light/14 h dark) conditions at 20°C (100 μE m^{-2} s^{-1}). For the expression studies, non-vernalized plants were grown in a growth chamber under long day or short day conditions at 20°C (100 μE m^{-2} s^{-1}).

Examination of heading-time Nineteen to 28 plants of each line were grown under each of the day length conditions. The number of days from unfolding of the first leaf to flag leaf unfolding (D1f) was scored for each plant. The D1fs were used as a measure of "days to heading". The difference of D1f between *Ppd-1a*-NIL and *Ppd-1b*-NIL were statistically analyzed by ANOVA. Photoperiod sensitivity was estimated as the ratios of the mean D1fs of plants under continuous light or LD conditions to those under SD conditions. The method for estimation of photoperiod sensitivity was originally described by Kato and Yamashita (1991), where D0f (days from sprouting to flag leaf unfolding) was used instead of D1f.

Estimation of growth phase Identification of the growth stage in the wheat plants was based on leaf stage. For example, the 2-leaf stage is the growth stage at which the second leaf has just unfolded, so that the plants have unfolded first and second leaves and a folded third leaf. Phase transition from vegetative to reproductive growth was defined as the time when the first node became visible and started to elongate.

Real-time PCR analysis Expression analyses were performed using RNAs extracted from leaves of three biological replicates each of one plant at each growth stage. The leaves were collected from plants at the 1-leaf to flag-leaf stages. Newly unfolded leaves were sampled from each plant: the first leaf from 1-leaf stage plants, the second leaf from 2-leaf stage plants, and the flag leaf from flag-leaf stage plants. The leaves were collected one hour after the beginning of the light period for the analysis of *VRN1* and *VRN3* expression, and three hours before the beginning of the light period for *WCO1* and *TaHd1*. All these genes are known to show diurnal expression patterns (Shimada et al., 2009) and the sampling times were aimed at collecting leaves at approximately the times of peak expression. Total RNAs were extracted using ISOGEN (Nippon-gene, Japan) and cDNAs were subsequently synthesized with oligo-dT primer in accordance with the protocol for the Ready-To-Go T-Primed First-Strand Kit (GE Healthcare Life Sciences). Real-time PCR analyses were performed using a LightCycler 2.0 (Roche Diagnostics GmbH) with gene specific primer sets for *VRN1*, *VRN3*, *WCO1* and *TaHd1*. The relative quantities of transcripts were determined by comparison to SYBR Green fluorescence of the *Ubiquitin* gene. For comparison of expression levels between *WCO1* and *TaHd1*, we examined the amplification efficiency of each primer set using four 2-fold gene-specific plasmid dilutions and compared their amplification efficiency each other. Expression level of *TaHd1* was rectified against that of *WCO1*, and then normalized by *Ubiquitin* gene. The sequences of the primer sets, the annealing temperatures, and the extension times for PCR are shown in Table 1.

RESULTS

Difference in heading times between *Ppd-1a*-NIL and *Ppd-1b*-NIL plants We previously reported that the near-isogenic line (NIL) of bread wheat cv. Triple Dirk carrying the photoperiod-insensitive *Ppd-1a* allele (*Ppd-1a*-NIL) exhibits earlier heading than the NIL with the photoperiod-sensitive *Ppd-1b* allele (*Ppd-1b*-NIL) under short day (SD) conditions (Murai et al., 2003). Here, we first re-examined heading times in the two NILs under three different light regimes (Table 2). Under continuous light, no significant difference in heading time was found between *Ppd-1a*-NIL and *Ppd-1b*-NIL; both genotypes showed early heading. Under LD conditions

Table 2. Days to heading (mean ± SE) under short day (SD, 10 h light), long day (LD, 16 h light) and continuous light (24 h light) conditions

NILs	SD	LD			
	10 h light (A)	16 h light (B)	24 h light (C)	(A)/(B)	(A)/(C)
Ppd-1a	67.1 ± 0.7	43.6 ± 0.2	35.8 ± 0.2	1.54	1.87
Ppd-1b	109.1 ± 1.0	46.8 ± 0.3	35.2 ± 0.4	2.33	3.10
F-value	1039.1**	71.8**	2.1^{ns}		

** : Significant difference at the 1% level with ANOVA.
^{ns} : Not significant.

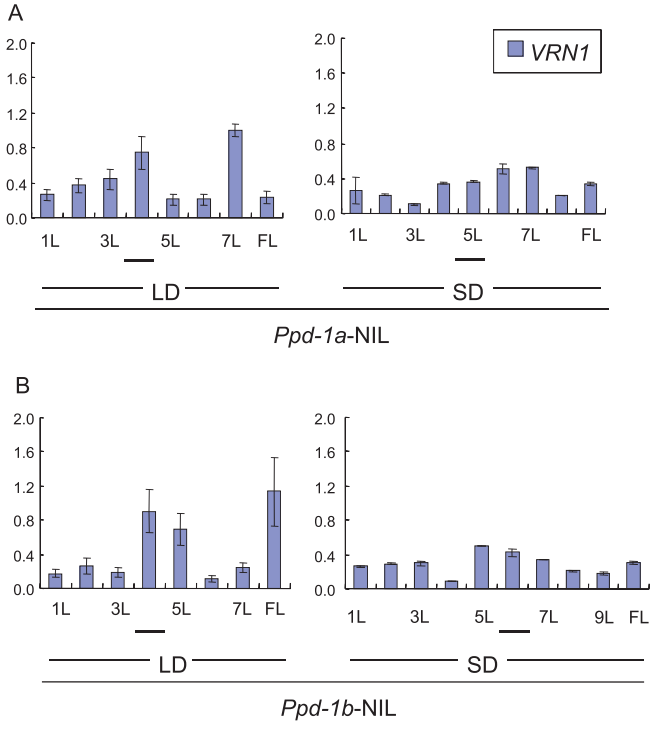

Fig. 1. *VRN1* expression levels during vegetative and reproductive growth stages. The growth stages are defined by leaf stage, from the 1-leaf stage (1L) to the flag-leaf stage (FL). Horizontal bars below the leaf stages indicate the time of transition from vegetative to reproductive growth. Expression levels are normalized and relative to the *Ubiquitin* gene. Error bats represent standard error. (A) Expression levels in photoperiod-insensitive *Ppd-1a*-NIL plants grown under long day (LD) or short day (SD) conditions. (B) Expression levels in photoperiod-sensitive *Ppd-1b*-NIL plants grown under long day (LD) or short day (SD) conditions.

(16 h light/8 h dark), heading time in *Ppd-1a*-NIL was significantly shorter than in *Ppd-1b*-NIL, although the difference was only three days on average. Under SD conditions, both NILs showed delayed heading but the effect was much greater in the *Ppd-1b*-NIL than in *Ppd-1a*-NIL. *Ppd-1a*-NIL plants transited from the vegetative to reproductive growth phase at the 4-leaf stage under LD conditions and at the 5-leaf stage under SD conditions. In comparison, *Ppd-1b*-NIL plants transited from the vegetative to reproductive growth phase at the 4-leaf stage under LD conditions, and at the 6-leaf stage under SD conditions (Figs. 1–3).

Similar *VRN1* expression patterns in the two NILs We used real-time PCR to compare the levels of *VRN1* expression in the two NILs during the vegetative and reproductive growth stages (Fig. 1). The cDNAs were obtained from leaves of 1-leaf to flag-leaf stage non-vernalized plants. Under LD conditions, both NILs transited from the vegetative to reproductive phase at the 4-leaf stage, and showed similar *VRN1* expression patterns. Expression of *VRN1* was low at the 1-leaf stage and increased as vegetative growth progressed. A higher level of expression was observed at the 4-leaf stage when the plants transited from the vegetative to reproductive phase. *VRN1* expression was low at the beginning of the reproductive phase and increased as reproductive growth progressed. Under SD conditions, a low level of *VRN1* expression was maintained during the vegetative to reproductive stages in both NILs. Despite the similar levels of *VRN1* expression, the timing of phase transition differed between *Ppd-1a*-NIL and *Ppd-1b*-NIL plants under SD conditions: the *Ppd-1a*-NIL transited to the reproductive growth phase at the 5-leaf stage, while the *Ppd-1b*-NIL transited at the 6-leaf stage. The occurrence of similar patterns of *VRN1* expression in *Ppd-1a*-NIL and *Ppd-1b*-NIL plants suggests that *VRN1* is not regulated by the photoperiod pathway controlled by *Ppd-1*.

***VRN3/WFT* expression under SD conditions differs in *Ppd-1a*-NIL and *Ppd-1b*-NIL plants** The expression of *VRN3/WFT* was similar in the two NILs, with abundant transcripts during the vegetative to reproductive stages (Fig. 2). Under LD conditions, expression showed two peaks: the first occurred at the 4-leaf stage when the plants transited to the reproductive phase; the second peak occurred at the 7-leaf stage in *Ppd-1a*-NIL and at the flag-leaf stage in *Ppd-1b*-NIL. By contrast, the pattern of *VRN3/WFT* expression differed considerably under SD conditions between the two NILs. *VRN3/WFT* was highly expressed at the 5-leaf stage in *Ppd-1a*-NIL plants when they transited from the vegetative to reproductive phase, but a very low level of expression was present in *Ppd-1b*-NIL plants. Thus, the *Ppd-1b* allele down-

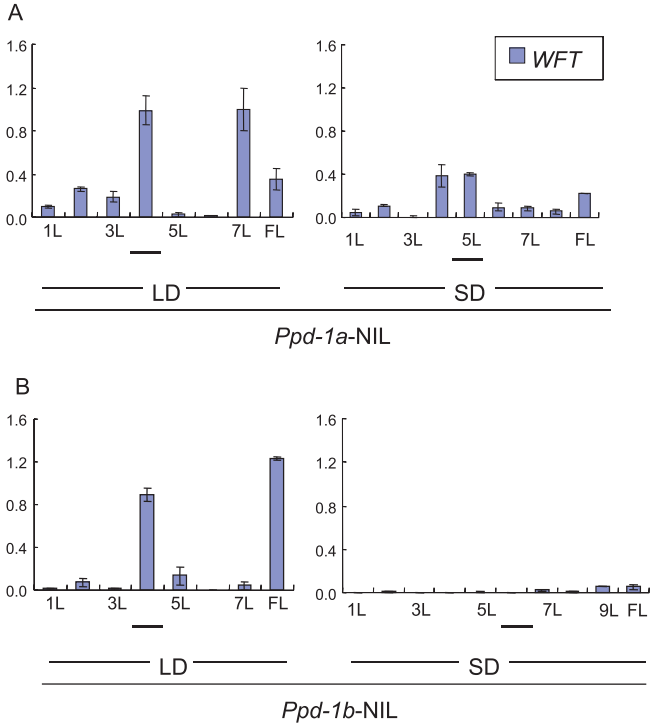

Fig. 2. *VRN3/WFT* expression levels during the vegetative and reproductive growth stages. The growth stages are defined by leaf stage, from the 1-leaf stage (1L) to flag-leaf stage (FL). Horizontal bars below the leaf stages indicate the time of transition from vegetative to reproductive growth. Expression levels are normalized and relative to the *Ubiquitin* gene. Error bats represent standard error. (A) Expression levels in photoperiod-insensitive *Ppd-1a*-NIL plants grown under long day (LD) or short day (SD) conditions. (B) Expression levels in photoperiod-sensitive *Ppd-1b*-NIL plants grown under long day (LD) or short day (SD) conditions.

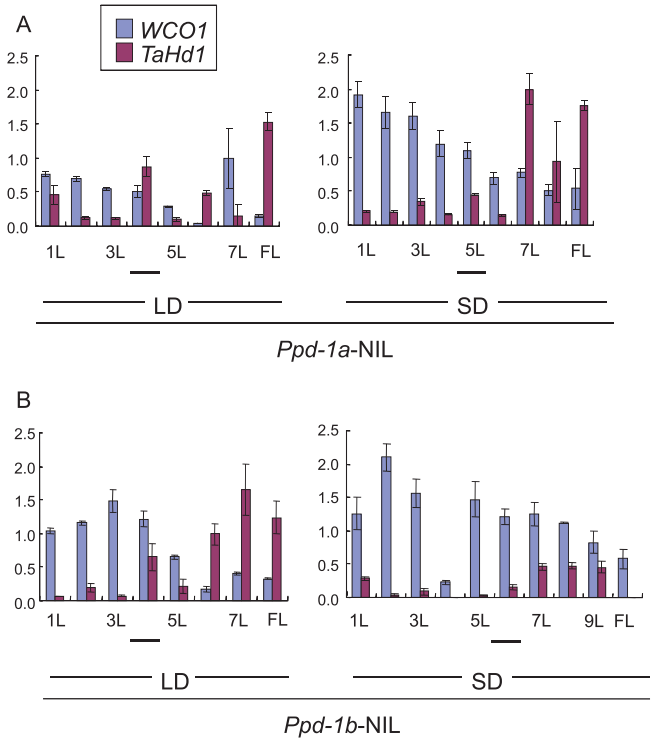

Fig. 3. Levels of *WCO1* (blue) and *TaHd1* (red) expression during the vegetative and reproductive growth stages. The growth stages are defined by leaf stage, from the 1-leaf stage (1L) to flag-leaf stage (FL). Horizontal bars below the leaf stages indicate the time of transition from vegetative to reproductive growth. Expression levels are normalized and relative to the *Ubiquitin* gene. Error bats represent standard error. (A) *WCO1* and *TaHd1* expression levels in photoperiod-insensitive *Ppd-1a*-NIL plants grown under long day (LD) or short day (SD) conditions. (B) *WCO1* and *TaHd1* expression levels in photoperiod-sensitive *Ppd-1b*-NIL plants grown under long day (LD) or short day (SD) conditions.

regulated *VRN3/WFT* expression under SD conditions.

The expression patterns of *WCO1* and *TaHd1* vary with *Ppd-1* allele Under LD conditions, *WCO1* expression was at a relatively higher level than that of *TaHd1* in both NILs during the vegetative growth stage; *TaHd1* expression increased as reproductive growth progressed (Fig. 3). Under SD conditions, *WCO1* was highly expressed in the vegetative stage but expression decreased during the phase transition and reproductive growth stages in both *Ppd-1a*-NIL and *Ppd-1b*-NIL. The level of *WCO1* expression in the vegetative stage under SD conditions was much higher than that under LD conditions, suggesting that *WCO1* is associated with vegetative growth under SD conditions, possibly through suppression of the phase transition. By contrast to *WCO1*, *TaHd1* was up-regulated during the reproductive stage. The level of *TaHd1* expression was much higher in *Ppd-1a*-NIL than *Ppd-1b*-NIL plants, suggesting that the *Ppd-1b* allele down-regulates *TaHd1* under SD conditions. The lower level of *TaHd1* expression might be associated with the delayed heading time in *Ppd-1b*-NIL plants.

DISCUSSION

In temperate cereals, the *Ppd-1* gene is the main regulator of photoperiod (day length) sensitivity in flowering. *VRN1* is up-regulated by a long photoperiod (Murai et al., 2003; Danyluk et al., 2003; Dubcovsky et al., 2006; Sasani et al., 2009), and, moreover, shows a diurnal pattern of expression under both LD and SD conditions. Expression of *VRN1* peaks at the beginning of the light period under both photoperiod conditions (Shimada et al., 2009). These observations suggest that *VRN1* expression is controlled by photoperiod. To examine the relationship between *Ppd-1* and *VRN1*, we examined *VRN1* expression patterns in NILs carrying either the photoperiod-insensitive *Ppd-1a* allele (*Ppd-1a*-NIL) or the photoperiod-sensitive *Ppd-1b* allele (*Ppd-1b*-NIL). We found that *VRN1* expression did not vary in plants with different *Ppd-1* alleles (Fig. 1), suggesting that expression of

this gene is not regulated by the photoperiod pathway mediated by *Ppd-1*. The flowering repressor gene, *VRN2,* reportedly shows a diurnal expression pattern under LD conditions (Dubcovsky et al., 2006). It is possible that *VRN1* expression is directly or indirectly affected by *VRN2* expression (Shimada et al., 2009; Distelfeld et al., 2009; Trevaskis, 2010). In barley, analysis of a F_2 population segregating for *Ppd-H1* alleles indicated that variation at *Ppd-H1* did not affect *VRN-H1/HvVRN1* expression (Campoli et al., 2012). This supports the conclusion that *VRN1* is not downstream of *Ppd-1*.

Comparison of the expression patterns of *VRN1* and *VRN3/WFT* under LD conditions showed that the *VRN3/WFT* expression pattern was correlated with that of *VRN1*. As a consequence, *VRN3/WFT* transcripts accumulated following the up-regulation of *VRN1* in both the *Ppd-1a*-NIL and *Ppd-1b*-NIL (Figs. 1 and 2). The fact that this occurs in both genotypes indicates that the association of *VRN3/WFT* expression with *VRN1* expression under LD conditions is not influenced by *Ppd-1*. Our observations support the idea that *VRN1* either directly or indirectly up-regulates *VRN3/WFT* (Shimada et al., 2009; Trevaskis, 2010).

The expression of *VRN3/WFT* was similar in *Ppd-1b*-NIL and *Ppd-1a*-NIL plants under LD conditions. However, under SD conditions, *Ppd-1b*-NIL plants had a very low level of *VRN3/WFT* expression, whereas *Ppd-1a*-NIL plants had a moderately high level of expression. Recently, Shaw et al. (2012) also reported that *Ppd-1b* allele down-regulates *VRN3/WFT* (identical with *TaFT1* in their study) using spring wheat cv. Paragon NILs. These results, together with the present flowering-time data (Table 2), strongly indicate that *Ppd-1b* down-regulates *VRN3/WFT*; alternatively, *Ppd-1a* up-regulates *VRN3/WFT* under SD conditions, resulting in delayed heading-time in *Ppd-1b*-NIL compared with *Ppd-1a*-NIL. Beales et al. (2007) reported that in vernalized seedlings of a photoperiod-insensitive wheat cv. Mercia NIL, the *Ppd-1a* allele was associated with high levels of *VRN3/WFT* expression. In this study, we used non-vernalized plants to avoid any possible effects of treatment on *VRN3/WFT* expression. Furthermore, we examined expression levels throughout the vegetative to reproductive growth stages in this study, contrary to the previous study (Beales et al., 2007; Shaw et al., 2012), in which expression analysis was performed in plants at seedling stage. We found that peak expression was at the 5-leaf stage in *Ppd-1a*-NIL under SD conditions, at the time when the plants are transiting from the vegetative to reproductive stages. Our observations indicate that up-regulation of *VRN3/WFT* confers the early heading phenotype in the *Ppd-1a*-NIL compared to the *Ppd-1b*-NIL.

Although *Ppd-1b*-NIL plants had low levels of *VRN3/WFT* expression in all growth stages, they initiated reproductive growth at the 6-leaf stage. It is not clear what mechanism is responsible for the eventual induction of reproductive growth in *Pdp-1b*-NIL plants under SD conditions, despite their low levels of expression of *VRN3/WFT*. The rice genome contains thirteen *FT*-like genes (Chardon and Damerval, 2005). One of these, *RICE FLOWERING LOCUS 1* (*RFT1*), plays a compensatory role in phase transition in plants with RNAi mediated knockdown of *Hd3a*, the rice *FT* ortholog (Komiya et al., 2008). Furthermore, analyses of plants with RNAi mediated knockdown of *RFT1* revealed that this gene regulates flowering in rice under LD conditions, which is not normally the photoperiod in which flowering occurs in this short day species (Komiya et al., 2009). With regard to temperate cereals, the barley genome contains five *FT*-like genes, one of which, *HvFT3*, is highly expressed under SD conditions (Faure et al., 2007). It is therefore possible that an *FT*-like gene other than *VRN3/WFT* is involved in the phase transition of *Ppd-1b*-NIL plants under SD conditions in wheat.

In barley, *VRN-H1/HvVRN1*, *HvFT1*, and *Ppd-H1* have been identified as orthologs of wheat *VRN1*, *VRN3*, and *Ppd-1* respectively. Hemming et al. (2008) reported that *VRN-H1/HvVRN1* and *HvFT1* were up-regulated by vernalization in doubled haploid lines regardless of *Ppd-H1* genotype. This suggests that the vernalization pathway involving *VRN1* and *VRN3* is independent of the photoperiod pathway of *Ppd-1*. These results support our interpretation that the interaction between *VRN1* and *VRN3* is not mediated by the photoperiod pathway related to *Ppd-1*.

The sequence similarity of *Ppd-1* to *Arabidopsis PRR7* has led to the suggestion that *Ppd-1* may function in a similar manner in the circadian clock, and act upstream of *FT* through *CO* (Shimada et al., 2009). In our earlier study (Shimada et al., 2009), we identified wheat *CO* (*WCO1*) by PCR using primers based on the sequence of barley *HvCO1*, which is located on chromosome 7H (Griffiths et al., 2003). Nine *CO* homologs have been identified in the barley genome. On the basis of sequence similarity and chromosomal synteny, *HvCO1* may be the counterpart of rice *Hd1*. A mapping study showed that *WCO1* was located on homoeologous group 7 (7A, 7B and 7D), indicating an orthologous relationship between *WCO1* and *HvCO1*. Wheat chromosomes 7A, 7B and 7D are syntenic to rice chromosome 6 (the location of the *Hd1* gene), suggesting that *WCO1* and *HvCO1* are orthologs of *Hd1*. It is known that *TaHd1*, previously identified as a wheat *CO*-like gene, is located on group 6 homoeologous chromosomes (Nemoto et al., 2003), indicating that *TaHd1* is not an ortholog of *Hd1*. Nevertheless, an analysis in transgenic rice indicated that *TaHd1* has some functions in flowering (Nemoto et al., 2003). Our previous diurnal expression studies using plants at the 3-leaf stage showed that *WCO1* and *TaHd1* had similar expression patterns (Shimada et al., 2009). Under LD condi-

tions, *WCO1* and *TaHd1* mRNAs accumulate during the dark period; *VRN3/WFT* mRNA accumulates from the beginning of the light phase in the *Ppd-1a*-NIL. Under SD conditions, *WCO1* and *TaHd1* expression showed similar patterns as under LD conditions. In the *Ppd-1b*-NIL, *WCO1* and *TaHd1* also showed similar expression patterns under LD conditions in leaves at the 3-leaf stage. However, under SD conditions, *WCO1* and *TaHd1* had lower expression during the dark period in the *Ppd-1b*-NIL compared with the *Ppd-1a*-NIL.

In this study, we examined heading time and phase transition timing in wheat NILs (Table 2, Figs. 1–3). Under SD conditions, *Ppd-1a*-NIL plants transited from the vegetative to reproductive phase around the 4-leaf stage, whereas *Ppd-1b*-NIL plants transited at the 6-leaf stage. To investigate the relationship between delayed phase transition in *Ppd-1b*-NIL under SD conditions and *CO* gene expression, we compared the patterns of expression of *WCO1* and *TaHd1* in *Ppd-1a*-NIL and the *Ppd-1b*-NIL plants (Fig. 3). Under SD conditions, *WCO1* was highly expressed in the vegetative stage; the level of expression decreased during phase transition and reproductive growth stages in both *Ppd-1a*-NIL and *Ppd-1b*-NIL plants. Higher expression level of *WCO1* in the early stages of *Ppd-1a* lines under SD conditions was also reported by Shaw et al. (2012). In the present study, we observed that the level of expression in the vegetative phase under SD conditions is much higher than under LD conditions, suggesting that *WCO1* functions in the suppression of phase transition under SD conditions. In barley, over-expression of *HvCO1* accelerated flowering time and caused up-regulation of *HvFT1* under LD conditions (Campoli et al., 2012). This suggests the possibility that under LD conditions *WCO1* activates *VRN3/WFT* while under SD conditions *WCO1* suppresses *VRN3/WFT*. This would parallel the case in short day plant rice, where *Hd1* (rice *CO*) suppresses *Hd3a* (rice *FT*) in flowering non-inducible LD conditions (Yano et al., 2000).

By contrast to *WCO1*, *TaHd1* is up-regulated during the reproductive stage in both *Ppd-1a*-NIL and *Ppd-1b*-NIL plants (Fig. 3). However, the expression level of *TaHd1* was much higher in *Ppd-1a*-NIL than *Ppd-1b*-NIL plants, suggesting that the *Ppd-1b* allele down-regulates *TaHd1* under SD conditions.

Conclusions The findings from the present study, together with those of previous studies, suggest a model of gene interaction for flowering as illustrated in Fig. 4. In this model, *VRN3/WFT* functions as an integrator of the *VRN1* related and *Ppd-1* related pathways. *VRN1* directly and/or indirectly up-regulates *VRN3/WFT*, resulting in phase transitions from vegetative to reproductive growth (Shimada et al., 2009; Trevaskis, 2010). Photoperiod (light-dark cycle) regulation of *VRN1* is probably induced directly and/or indirectly by *VRN2* expression (Shimada et al., 2009; Dubcovsky et al., 2006; Trevaskis, 2010). Under LD conditions, the effect of the photoperiod signal is not moderated by the influence of the *Ppd-1* allele on *WCO1* and *TaHd1* expression (Fig. 4, A and C). Under SD conditions, *WCO1* is up-regulated regardless of the *Ppd-1* allele (Fig. 4, B and D). In this model, *WCO1* shows inverse function in LD and SD; up-regulation of *VRN3/WFT* under LD conditions and down-regulation of *VRN3/WFT* under SD conditions. Therefore, the up-regulation of *WCO1* and the down-regulation of *VRN1* result in the down-regulation of *VRN3/WFT* and delayed flowering under SD conditions. Furthermore,

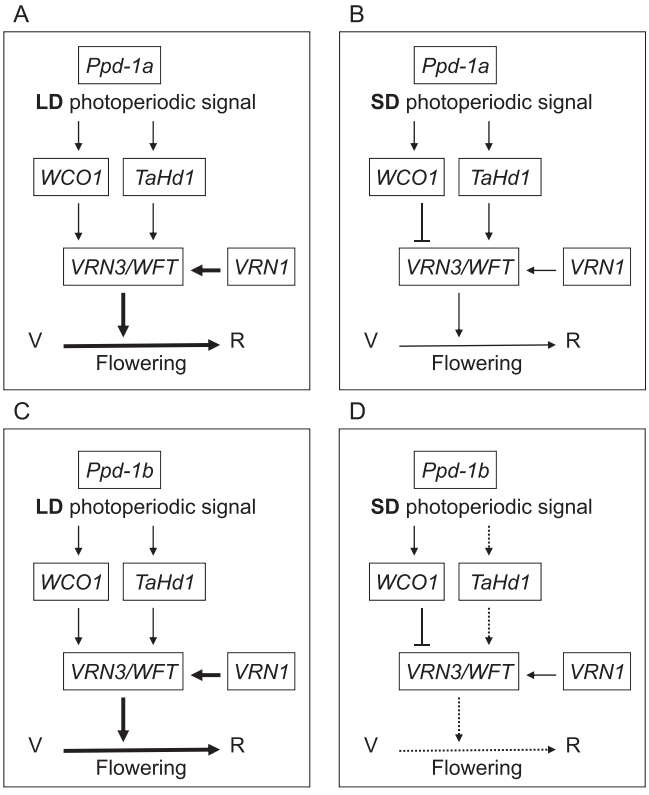

Fig. 4. Model for the interactions between the genetic pathways controlling flowering in wheat. *VRN3/WFT* acts as an integrator of all pathways. *VRN1* directly or indirectly induces *VRN3/WFT* expression, leading to flowering. The photoperiod signals involving *Ppd-1* are transmitted to *WCO1* and *TaHd1*, and regulate *VRN3/WFT* expression. The present study suggests that *WCO1* negatively regulates and *TaHd1* positively regulates *VRN3/WFT* expression under short day (SD) conditions. The *VRN3/WFT* protein is postulated to be a mobile florigen that induces floral meristem determination and promotes phase transition from vegetative (V) to reproductive (R) growth. Arrows and T-bars mean promotive and suppressive effect, respectively. (A and B) Flowering pathway interactions in photoperiod-insensitive *Ppd-1a*-NIL plants grown under long day (LD) or short day (SD) conditions. Arrows indicated by bold lines show stronger effects. (C and D) Flowering pathway interactions in photoperiod-insensitive *Ppd-1b*-NIL plants grown under long day (LD) or short day (SD) conditions. Arrows indicated by bold lines show stronger effects. Arrows indicated by dotted lines indicate weaker effects.

photoperiod signaling via the *Ppd-1b* allele affects *TaHd1* expression: *TaHd1* is down-regulated, resulting in down-regulation of *VRN3/WFT* (Fig. 4D). The up-regulation of *WCO1* and the down-regulation of *TaHd1* together with *VRN1* might strongly induce down-regulation of *VRN3/WFT*, resulting in an extreme delay in flowering in plants with the *Ppd-1b* allele.

We are grateful to the National Bioresource Project – Wheat (NBRP-KOMUGI) for providing wheat materials and the EST database. This work was supported in part by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 21380009) to K. Murai.

REFERENCES

Beales, J., Turner, A., Griffiths, S., Snape, J. W., and Laurie, D. A. (2007) A *Pseudo-Response Regulator* is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.). Theor. Appl. Genet. **115**, 721–733.

Campoli, C., Drosse, B., Searle, I., Coupland, G., and von Korff, M. (2012) Functional characterisation of *HvCO1*, the barley (*Hordeum vulgare*) flowering time ortholog of *CONSTANS*. Plant J. **69**, 868–880.

Chardon, F., and Damerval, C. (2005) Phylogenomic analysis of the PEBP gene family in cereals. J. Mol. Evol. **61**, 579–590.

Danyluk, J., Kane, N. A., Breton, G., Limin, A. E., Fowler, D. B., and Sarhan, F. (2003) *TaVRT-1*, a putative transcription factor associated with vegetative to reproductive transition in cereals. Plant Physiol. **132**, 1849–1860.

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

Dubcovsky, J., Loukoianov, A., Fu, D., Valarik, M., Sanchez, A., and Yan, L. (2006) Effect of photoperiod on the regulation of wheat vernalization genes *VRN1* and *VRN2*. Plant Mol. Biol. **60**, 469–480.

Faure, S., Higgins, J., Turner, A., and Laurie, D. A. (2007) The *FLOWERING LOCUS T*-like gene family in barley (*Hordeum vulgare*). Genetics **176**, 599–609.

Griffiths, S., Dunford, R. P., Coupland, G., and Laurie, D. A. (2003) The evolution of *CONSTANS*-like gene families in barley, rice and *Arabidopsis*. Plant Physiol. **131**, 1855–1867.

Hemming, M.N., Peacock, W.J., Dennis, E. S., and Trevaskis, B. (2008) Low-temperature and daylength cues are integrated to regulate *FLOWERING LOCUS T* in barley. Plant Physiol. **147**, 355–366.

Imaizumi, T., and Kay, S. A. (2006) Photoperiodic control of flowering: not only by coincidence. Trends Plant Sci. **11**, 550–558.

Kato, K., and Yamashita, S. (1991) Varietal variation in photoperiodic response, chilling requirement and narrow-sense earliness and their relation to heading time in wheat (*Triticum aestivum* L.). Jpn. J. Breed. **41**, 475–484.

Komiya, R., Ikegami, A., Tamaki, S., Yokoi, S., and Shimamoto, K. (2008) *Hd3a* and *RFT1* are essential for flowering in rice. Development **135**, 767–774.

Komiya, R., Yokoi, S., and Shimamoto, K. (2009) A gene network for long-day flowering activates *RFT1* encoding a mobile flowering signal in rice. Development **136**, 3443–3450.

Laurie, D. A. (1997) Comparative genetics of flowering time. Plant Mol. Biol. **35**, 167–177.

Law, C. N., Sutka, J., and Worland, A. J. (1978) A genetic study of day-length response in wheat. Heredity **41**, 185–191.

Murai, K., Miyamae, M., Kato, H., Takumi, S., and Ogihara, Y. (2003) *WAP1*, a wheat *APETALA1* homolog, plays a central role in the phase transition from vegetative to reproductive growth. Plant Cell Physiol. **44**, 1255–1265.

Nakamichi, N. (2011) Molecular mechanisms underlying the *Arabidopsis* circadian clock. Plant Cell Physiol. **52**, 1709–1718.

Nemoto, Y., Kisaka, M., Fuse, T., Yano, M., and Ogihara, Y. (2003) Characterization and functional analysis of three wheat genes with homology to the *CONSTANS* flowering time gene in transgenic rice. Plant J. **36**, 82–93.

Pugsley, A. T. (1968) Genetic studies of phasic development and their application to wheat breeding. In: Proceedings of the 3rd International Genetics Symposium, (eds.: K. W. Findlay, and K. W. Shepherd), pp. 288–293. Australian Academy of Science, Canberra.

Sasani, S., Hemming, M. N., Oliver, S. N., Greenup, A., Tavakkol-Afshari, R., Mahfoozi, S., Poustini, K., Sharifi, H.-R., Dennis, E. S., Peacock, W. J., and Trevaskis, B. (2009) The influence of vernalization and daylength on expression of flowering-time genes in the shoot apex and leaves of barley (*Hordeum vulgare*). J. Exp. Bot. **60**, 2169–2178.

Shaw, L. M., Turner, A. S., and Laurie, D. A. (2012) The impact of photoperiod insensitive *Ppd-1a* mutations on the photoperiod pathway across the three genomes of hexaploid wheat (*Triticum aestivum*). Plant J. DOI: 10.1111/j.1365-313X.2012.04971.x

Shimada, S., Ogawa, T., Kitagawa, S., Suzuki, T., Ikari, C., Shitsukawa, N., Abe, T., Kawahigashi, H., Kikuchi, R., Handa, H., and Murai, K. (2009) A genetic network of flowering time genes in wheat leaves, in which an *APETALA1/FRUITFULL*-like gene, *VRN1*, is upstream of *FLOWERING LOCUS T*. Plant J. **58**, 668–681.

Trevaskis, B. (2010) The central role of the *VERNALIZATION1* gene in the vernalization response of cereals. Funct. Plant Biol. **37**, 479–487.

Trevaskis, B., Bagnall, D. J., Ellis, M. H., Peacock, W. J., and Dennis, E. S. (2003) MADS box genes control vernalization-induced flowering in cereals. Proc. Natl. Acad. Sci. USA **100**, 13099–13104.

Turner, A., Beales, J., Faure, S., Dunford, R. P., and Laurie, D. A. (2005) The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. Science **310**, 1031–1034.

Wilhelm, E. P., Turner, A. S., and Laurie, D. A. (2009) Photoperiod insensitive *Ppd-A1a* mutations in tetraploid wheat (*Triticum durum* Desf.). Theor. Appl. Genet. **118**, 285–294.

Worland, T., and Snape, J. W. (2001) Genetic basis of worldwide wheat varietal improvement. In: The World Wheat Book, A History of Wheat Breeding, (eds.: A. P. Bonjean, and W. J. Angus), pp. 59–100. Lavoisier Publishing, Londres.

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

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., and Sasaki, T. (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. Plant Cell **12**, 2473–2484.