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RESEARCH PAPER

Effects of photo and thermo cycles on flowering time in barley: a genetical phenomics approach

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Abstract

The effects of synchronous photo (16 h daylength) and thermo (2 °C daily fluctuation) cycles on flowering time were compared with constant light and temperature treatments using two barley mapping populations derived from the facultative cultivar 'Dicktoo'. The 'Dicktoo'×'Morex' (spring) population (DM) segregates for functional differences in alleles of candidate genes for *VRN-H1*, *VRN-H3*, *PPD-H1*, and *PPD-H2*. The first two loci are associated with the vernalization response and the latter two with photoperiod sensitivity. The 'Dicktoo'×'Kompolti korai' (winter) population (DK) has a known functional polymorphism only at *VRN-H2*, a locus associated with vernalization sensitivity. Flowering time in both populations was accelerated when there was no fluctuating factor in the environment and was delayed to the greatest extent with the application of synchronous photo and thermo cycles. Alleles at *VRN-H1*, *VRN-H2*, *PPD-H1*, and *PPD-H2*—and their interactions—were found to be significant determinants of the increase/decrease in days to flower. Under synchronous photo and thermo cycles, plants with the Dicktoo (recessive) *VRN-H1* allele flowered significantly later than those with the Kompolti korai (recessive) or Morex (dominant) *VRN-H1* alleles. The Dicktoo *VRN-H1* allele, together with the late-flowering allele at *PPD-H1* and *PPD-H2*, led to the greatest delay. The application of synchronous photo and thermo cycles changed the epistatic interaction between *VRN-H2* and *VRN-H1*: plants with Dicktoo type *VRN-H1* flowered late, regardless of the allele phase at *VRN-H2*. Our results are novel in demonstrating the large effects of minor variations in environmental signals on flowering time: for example, a 2 °C thermo cycle caused a delay in flowering time of 70 d as compared to a constant temperature.

Key words: *BM5A*, *Hordeum vulgare*, *HvFT1*, *HvFT3*, *HvPRR7*, *ZCCT-H*.

Introduction

Flowering time is one of the most important adaptive characteristics of plants. Genetic regulation of physiological processes acts to ensure that flowering occurs at seasonal optima for pollination, fertilization, and seed development. In temperate zones, vernalization sensitivity and juvenility repress flowering early in plant development, allowing the plant to avoid freezing injury (Danyluk *et al*., 2003) and to achieve sufficient vegetative growth for supporting the high energy demands of flowering and seed set (Boss *et al*., 2004). Additional regulatory pathways are involved in perceiving and transferring environmental signals to floral meristem and organ identity genes (reviewed by Boss *et al*., 2004; Jack, 2004; Amasino, 2005). A better understanding of the genetic components of flowering time regulation will aid in the improvement of agricultural productivity in new production zones, or in conventional zones subject to greater climatological fluctuations.

The critical genes in the complex hierarchical cascades regulating flowering are best described in *Arabidopsis* (*Arabidopsis thaliana* L.) (reviewed by Boss *et al*., 2004; Amasino, 2005; Imaizumi and Kay, 2006). These genes are assigned roles in one of four pathways: photoperiod (including the perception of light quality and quantity); autonomous; vernalization; and hormonal. Signals from the pathways converge at a limited number of floral integrator genes, which in turn activate meristem identity

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genes. Information from *Arabidopsis* was an indispensable platform for rapidly isolating the homologues from cereal crops. These include: *PSEUDO RESPONSE REGULATOR 7* (*PRR7*) (Turner *et al*., 2005; Beales *et al*., 2007) and GIGANTEA (*GI*) (Dunford *et al*., 2005) in the circadian central oscillator (McClung, 2006); genes in the photoperiod regulation pathway, including photoreceptors (*PhyA*, *PhyB*, *PhyC*, *Cry1*, *Cry2*) (Szűcs *et al*., 2006) and the *CONSTANS* (*CO*) gene family (Griffiths *et al*., 2003); the *FLOWERING LOCUS T* (*FT*) family of integrator genes (Yan *et al*., 2006; Faure *et al*., 2007); and the meristem identity gene *AP1* (*BM5A* in barley) (Yan *et al*., 2003; Danyluk *et al*., 2003; von Zitzewitz *et al*., 2005). Interestingly, for some of these genes, for example, *GI*, *CO*, and photoreceptors (Griffiths *et al*., 2003; Dunford *et al*., 2005; Szűcs *et al*., 2006) there is no association between phenotypic variation in flowering time and allele sequence variation.

Attention has therefore focused on *Triticeae* genes that are significant determinants in flowering, and these can be broadly classified as *VRN* (vernalization response) or *PPD* (photoperiod sensitivity) genes (Cockram *et al*., 2007; Trevaskis *et al*., 2007). These *Triticeae* genes have different designations (and often functions) than in *Arabidopsis*. *Arabidopsis AP1* corresponds to the candidate gene of the *Triticeae VRN1* locus (Danyluk *et al*., 2003; Yan *et al*., 2003), *PRR7* to *PPD1* (Turner *et al*., 2005; Beales *et al*., 2007) and one member of the *FT* gene family (*FT1*), to *VRN3* (Yan *et al*., 2006; Faure *et al*., 2007). Another member of this family (*HvFT3*) is considered a candidate for the *PPD-H2* locus (Faure *et al*., 2007). In cereals, *ZCCT1 (ZCCT-H* in barley), with no known *Arabidopsis* homologue, was identified as the candidate gene of the *VRN2* locus (Yan *et al*., 2004; Dubcovsky *et al*., 2005). This gene seems to occupy an intermediate position between the vernalization and photoperiod pathways, as both low temperature vernalization and photoperiod influences its activity (Dubcovsky *et al*., 2006).

The *VRN* loci have been assigned roles in a general model of flowering in cereals (Yan *et al*., 2006; Cockram *et al*., 2007). Briefly, vernalization saturation represses the activity of the dominant *VRN2* allele, allowing expression of the recessive alleles at *VRN3* and *VRN1*. *VRN3* enhances activity of *VRN1* under a long photoperiod, resulting in earlier heading. The roles of the *PPD* loci within this scheme are not fully resolved. In barley, *PPD-H1* appears to affect flowering under long photoperiods, and *PPD-H2* under short ones (Laurie *et al*., 1995; Turner *et al*., 2005).

Genetic analyses of the flowering time phenotype in the *Triticeae* under greenhouse or field conditions are rendered complex by uncontrolled sources of variation. Controlled environment tests allow for trait dissection but usually a limited number of environmental cues are varied at a constrained number of levels. Vernalization treatment and photoperiod duration are the two most widely-studied cues in the *Triticeae* (reviewed by Cockram *et al*., 2007; Trevaskis *et al*., 2007). Usually, all other factors are held constant or their effects are ignored. The results of preliminary experiments have recently been reported, in which a daily fluctuation of 2 °C (18/16 °C day/night) versus a constant temperature (18 °C) treatment during plant growth dramatically altered flowering time in a reference panel of barley accessions (Karsai *et al*., 2008). Spring growth habit accessions were the least affected and facultative accessions were the most affected. These results underscore the need for rigorous characterization of all environmental cues in flowering time experiments. A deeper understanding of all environmental cues will be of assistance in developing tools to alter flowering time without manipulating vernalization or photoperiod.

In this study a 'genetical phenomics' approach was applied to understand the effects of fluctuating temperature on the phenotypic manifestations of five loci involved in flowering time of barley: *VRN-H1*, *VRN-H2*, *VRN-H3*, *PPD-H1*, and *PPD-H2*. This investigation was facilitated by the availability of (i) allele-specific primers for the candidate or putative genes of these loci, (ii) very well-characterized barley accessions representative of the three growth habit types, and (iii) two well-characterized doubled haploid (DH) mapping populations.

Materials and methods

Plant materials

The Morex (M, spring), Dicktoo (D, facultative), and Kompolti korai (K, winter) cultivars and the two DH mapping populations derived from the cross of D×M (DM) and D×K (DK) used for these experiments have been well characterized at the genotypic and phenotypic levels (Pan *et al*., 1994; Karsai *et al*., 2005, 2006, 2007, 2008; von Zitzewitz *et al*., 2005; Szűcs *et al*., 2006).

Phenotypic characterizations

Controlled environment experiments were carried out in the Phytotron facilities of the Agricultural Research Institute of HAS, Martonvásár, Hungary using Conviron PGV type growth chambers (Conviron Ltd., Winnipeg, Canada). The technical parameters of the growth chambers and control systems for temperature and light intensity, are detailed in Karsai *et al*. (2004). Three different growth conditions were applied for the two DH populations and the three parents for a total of 150 d: (i) continuous light and a constant temperature of 18 °C, (ii) 16/8 h light/dark per 24 h with a constant temperature of 18 °C, and (iii) 16/8 h light/dark per 24 h with a temperature cycle of 18 °C light/16 °C dark, applied synchronously with the photo cycle. The two latter growth conditions were applied in phytotron chambers, while the continuous light and constant temperature conditions were applied in a phytotron chamber and in a greenhouse for the DK and the DM populations, respectively. In the greenhouse, a small natural daily variation in light intensity and in temperature could not be excluded completely. Acronyms for the three growth conditions applied to each of the two populations are as follows: 24C, 16C, and 16T, where the number refers to the number of h of light/24 h (24 or 16), and C or

T refer to the temperature treatment, constant or thermo cycle. Flowering time (FT) was recorded as the number of days elapsed between planting and flowering and referred as FT24C, FT16C, and FT16T based on the growth conditions applied. Germplasm that did not flower was assigned an FT value of 150. The same light intensity (photosynthetic photon flux density (PPFD) of 220 μmol m^{-2} s^{-1}) provided by a mixture of fluorescent tubes (Sylvania cool white) and incandescent lamps was used under all three growth conditions. Relative humidity was also maintained at the same level of ~80% under the treatments. In the case of the DM (facultative×spring) population, the DH lines and parents were grown without vernalization. In the case of the DK (facultative×winter) population, all plant material was vernalized for 6 weeks at 3 °C with a 9/15 h light/dark photoperiod regime at low light intensity (10 μmol m^{-2} s^{-1}). Each genotype was replicated twice within each growth condition, giving an average plant density of approximately 60 plants m^{-2}.

Genotyping, linkage map construction, and QTL analysis

The DM linkage map consists of 165 loci of various types (e.g. AFLP, RFLP, SSR, STS, and ASGTs (allele-specific gene tags) with a total recombination length of 1040 cM and an average marker spacing of 6.3 cM (Pan *et al*., 1994; Skinner *et al*., 2006; Szűcs *et al*., 2006). The DK linkage map consists of 236 loci of various types, with a total recombination length of 1107 cM and an average marker distance of 4.5 cM (Karsai *et al*., 2005, 2007; Szűcs *et al*., 2006). The *VRN-H1* and *VRN-H2* loci were mapped with allele-specific primers of the respective candidate genes *BM5A* and *ZCCT-H* in the DM and DK populations (von Zitzewitz *et al*., 2005; Karsai *et al*., 2005).

For this experiment, the *VRN-H3* and the *PPD-H1* loci were mapped in the DM population as codominant CAPS markers designed for *HvFT1* (Yan *et al*., 2006) and for *HvPRR7* (Turner *et al*., 2005), respectively. The *PPD-H2* locus was mapped as a dominant marker based on the presence/absence of the *HvFT3* gene as described by Faure *et al*. (2007). In the DK population two SNPs were identified in the aligned *HvFT1* (*VRN-H3*) sequences of Dicktoo (EU007827) and Kompolti korai (EU007828). The exon 3 SNP was utilized as the basis for a CAPS marker employing the primer pairs of HvFT1.03F (5′-CTT GCT CCC TCA TAC CCT AG-3′) and HvFT1.04R (5′-GCT TAA TTC GTG GCT GGC TTC-3′) and product digestion with *BsrDI*. The *PPD-H1* and *PPD-H2* loci were not mapped in DK because Dicktoo and Kompolti korai are monomorphic for the tested CCT domain SNP [SNP22 based on Turner *et al*. (2005)] in *HvPRR7* and both accessions have the deletion of *HvFT3*. Linkage maps were constructed using JoinMap 4.0 (van Ooijen, 2006). QTL analyses were performed using composite interval mapping (CIM) Model 6, with forward regression and backward elimination as implemented in WinQTL Cartographer v. 2.5 (Wang *et al*., 2007). Threshold levels were set using 500 permutations.

Results

Phenotypic and genotypic characterization of the parental lines

Flowering was most accelerated in Morex, Dicktoo, and Kompolti korai when there were no thermo or photo fluctuations (24C) (Table 1). Flowering was delayed by the application of a daily photo cycle at a constant temperature (16C) but simultaneous photo and thermo cycling (16T) had the greatest effect on delaying flowering (Table 1). Morex was the least sensitive, while Dicktoo the most sensitive, to the fluctuating factors.

Allele-specific primers revealed diversity at *VRN* and *PPD* loci in the parental lines (Table 2). In the DM population, the effect of the *VRN-H1*, *VRN-H3*, *PPD-H1*, and *PPD-H2* loci could be evaluated based on the functional allele differences between the two parental lines in the respective candidate genes. In the DK population, there was only functional segregation in the candidate gene of the *VRN-H2* locus. The minor polymorphisms in the candidate genes for the *VRN-H1* and *VRN-H3* loci are not known to affect phenotype, but these variations allowed us to monitor allelic segregation at these loci. In the remainder of this report, the first letter of the parental cultivar's name will be used to refer to the allele phases in each individual *VRN-H* and *PPD-H* locus.

Effects of photo and thermo fluctuations on flowering in DM

There were similar patterns of flowering time amongst parents and DH lines under the three growth conditions (correlations 0.79–0.88, see Supplementary Fig. S1 at *JXB* online). As shown in Table 1, the population as a whole was earliest to flower under 24C, but this mean value was not significantly different from that for 16C. Flowering was significantly delayed under 16T and eight DH lines did not flower in 150 d. QTL analyses revealed that *PPD-H1* and *VRN-H1* were the most significant determinants of

Table 1. *Flowering characteristics of the Dicktoo (facultative)×Morex (spring) (DM) and the Dicktoo (facultative)×Kompolti korai (winter) (DK) barley doubled haploid mapping populations under various environmental conditions*

Growth condition[a]	Dicktoo	Morex	DM population			Dicktoo	Kompolti	DK population		
			Mean[b]	Interval	LSD			Mean[b]	Interval	LSD
24C[c]	42	38	42 a	25–78	3.0	28	53	42 a	26–80	3.5
16C	37	40	44 a	27–95	4.0	39	61	52 b	31–94	5.1
16T	128	54	74 c	39–150	4.8	109	74	77 c	43–104	6.6

[a] 24C, continuous light and constant temperature; 16C, 16 h photoperiod and constant temperature; 16T, 16 h photoperiod and thermo cycle.
[b] Mean values followed by the same letter within column are not significantly different from each other at the $P=0.05$ level.
[c] Values for Dicktoo, Morex, and the DM population are from Pan *et al*., 1994. See Materials and methods for details.

the observed phenotypic variation in flowering time under the three growth conditions (Table 3; see Supplementary Fig. S2 at *JXB* online). The M allele at *PPD-H1* and the D allele at *VRN-H1* delayed flowering. The additive effect of the M allele at *PPD-H1* was to delay flowering for 12 d and 13 d under 16C and 16T, respectively. The difference in additive effect of the D allele at *VRN-H1* was much greater under the 16T condition (9 d and 24 d under 16C and 16T, respectively). Of the two genes, *VRN-H1* determined a greater proportion of the phenotypic variance at 24C and 16T and *PPD-H1* at 16C.

The combined effects of *VRN-H1* and *PPD-H1* explained most of the phenotypic variation in the experiment (two-locus R^2 values were 81.4%, 83.5%, and 74.0% for FT24C, FT16C, and FT16T, respectively). As a result, the mean flowering times of lines with the parental allele combinations at the two loci were statistically the same as the respective parents under 24C and 16C. At 16T, however, the DD (*VRN-H1/PPD-H1*) lines headed significantly earlier than Dicktoo (84 d versus 128 d, respectively), while the MM lines were significantly later than Morex (66 d versus 54 d, respectively). The non-parental allele combinations were responsible for the significant phenotypic transgressive segregation shown in Fig. S1 in Supplementary data at *JXB* online. Lines with MD alleles at *VRN-H1/PPD-H1* flowered significantly earlier, while lines with DM alleles at *VRN-H1/PPD-H1* flowered significantly later than the parents and parental allele combinations. In addition, flowering of the non-parental combinations were significantly influenced by the application of a thermo cycle. The MD (*VRN-H1/PPD-H1*) was the only subclass with a relatively uniform reaction to the thermo cycle. Conversely, the largest scattering was observed in the DM (*VRN-H1/PPD-H1*) subclass (Fig. 1).

PPD-H2 had a significant main effect only under 16T, where it explained 8% of the phenotypic variance (Table 3; see Supplementary Fig. S2 at *JXB* online). This locus had a greater effect on flowering time, however, in association with certain alleles at other loci (Fig. 2). Considering the four possible *VRN-H1* and *PPD-H1* allele combinations, the allele phase at *PPD-H2* had the most pronounced effect when the late flowering (M) allele at *PPD-H1* was juxtaposed with the winter (D) allele at

Table 2. *Allele compositions of three barley parental lines at vernalization response (VRN) and photoperiod sensitivity (PPD) loci based on polymorphisms associated with the candidate or putative gene functions of each locus*

Locus/gene	Chromosome	Site of functional polymorphism	Allele types of the varieties[a]		
			Dicktoo (facultative)	Morex (spring)	Kompolti (winter)
VRN-H1/BM5A	5H	Intron 1 deletion (1)	Winter	Spring	Winter
VRN-H2/ZCCT-H	4H	Presence/absence (2)	Spring	Spring	Winter
VRN-H3/HvFT1	7H	SNP haplotype in intron 1 (3)	Winter	Spring	Winter
PPD-H1/HvPRR7	2H	SNP in the CCT domain (4)	Early flowering under long days	Late flowering under long days	Early flowering under long days
PPD-H2/HvFT3	1H	Presence/absence (5)	Late flowering under short days	Early flowering under short days	Late flowering under short days

[a] Primers used for detection and the allele nomenclatures are based on the relevant references: (1) von Zitzewitz *et al.* (2005); (2) Karsai *et al.* (2005); (3) Yan *et al.* (2006); (4) Turner *et al.* (2005); (5) Faure *et al.* (2007).

Table 3. *Effects of the vernalization response (VRN) and photoperiod sensitivity (PPD) loci on flowering time in two barley mapping populations under various environmental conditions*

Growth condition[a]	*VRN-H1*			*VRN-H2*			*VRN-H3*			*PPD-H1*			*PPD-H2*		
	LOD	R^2 (%)	Add. eff.	LOD	R^2 (%)	Add. eff.	LOD	R^2 (%)	Add. eff.	LOD	R^2 (%)	Add. eff.	LOD	R^2 (%)	Add. eff.
Dicktoo×Morex															
24C	32.1	49.0	11		–			ns		23.5	40.0	–9		ns	
16C	22.7	30.3	9		–			ns		31.2	55.0	–12		ns	
16T	22.3	41.6	24		–			ns		12.0	17.2	–13	7	7.8	9
Dicktoo×Kompolti korai															
24C	4.6	2.1	2	50.1	77.8	–12		ns			–			–	
16C	3.7	3.2	3	34.5	63.8	–12		ns			–			–	
16T	24.6	49.5	12	11.0	15.5	–7		ns			–			–	

[a] 24C, continuous light and constant temperature; 16C, 16 h photoperiod and constant temperature; 16T, 16 h photoperiod and thermo cycle.

VRN-H1 under 16T. In this case, the allele phase at *PPD-H2* explained 83% of the phenotypic variance and the Dicktoo allele (lack of the *HvFT3* gene) resulted in extremely delayed flowering; eight DH lines of the 10 carrying this combination did not flower (Fig. 1). There was only one combination of *PPD-H1* and *VRN-H1* alleles where the allele at *PPD-H2* had no effect at all on flowering and that was the early flowering (D) allele at *PPD-H1* configured with the spring (M) allele at *VRN-H1*.

No QTL main effect was detected at *VRN-H3* (Table 3). The effect of *VRN-H3* and the three-way loci interactions with the *VRN-H1* and *PPD-H1* loci could not be discerned due to segregation distortion in two of the four allele classes at *VRN-H1* and *PPD-H1*.

Effects of photo and thermo fluctuations on flowering in DK

In DK, the average flowering time of the population was also the earliest under 24C (Table 1). Varying photoperiod duration and holding temperature constant (16C) significantly delayed the average flowering of the population (10 d). The delay was relatively uniform: the correlation was 0.92 between FT24C and FT16C (see Supplementary Fig. S1 at *JXB* online). The combined effects of light and thermo cycles further delayed the mean flowering time of the population by 25 d. Individual line responses were not as consistent: the correlations between FT16T and the other two growth conditions were both 0.52 (see Supplementary Fig. S1 at *JXB* online). Even under 16T all lines flowered within 110 d.

VRN-H2 had a very large effect on flowering time at 24C and 16C, accounting for 78% and 64% of the phenotypic variance, respectively (Table 3, see Supplementary Fig. S2 at *JXB* online). The winter (K) allele main additive effect was a 12 d delay in flowering. The *VRN-H1* locus had a significant effect under these conditions, but it explained a very low portion of the phenotypic variance. The application of synchronous light and thermo cycles resulted in a shift in the significance of the effects of these two loci: at 16T *VRN-H1* explained close to 50% of the phenotypic variance and *VRN-H2* only 16%.

VRN-H2 and *VRN-H1* jointly accounted for most of the phenotypic variation, irrespective of growth condition: the two-locus R^2 values were 0.91 for FT24C, 0.83 for FT16C, and 0.69 for FT16T. The average flowering times of lines with parental allele combinations at these two loci were statistically the same as the respective parents under all three conditions, with one exception. At 16T, the average flowering of the DD lines was again significantly earlier than that of Dicktoo (86 d versus 109 d). As shown in Fig. 3, there is a pattern of growth condition-dependent epistasis between these two loci. Two features are noteworthy. First, the K allele at *VRN-H1* always resulted in significantly earlier flowering than the D allele, regardless of growth condition or allele phase of *VRN-H2*. Second, the winter allele (K) at *VRN-H2* delayed

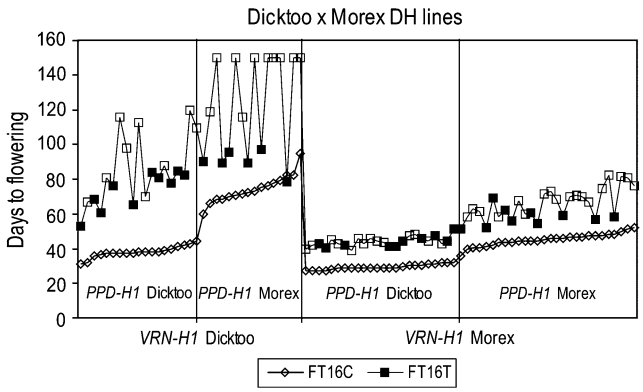

Fig. 1. Effect of synchronous photo and thermo cycles on the major developmental genes in the Dicktoo (facultative)×Morex (spring) barley population. Within the graph of FT16T, the empty square represents the Dicktoo null allele in *PPD-H2*, while the full square stands for the Morex allele.

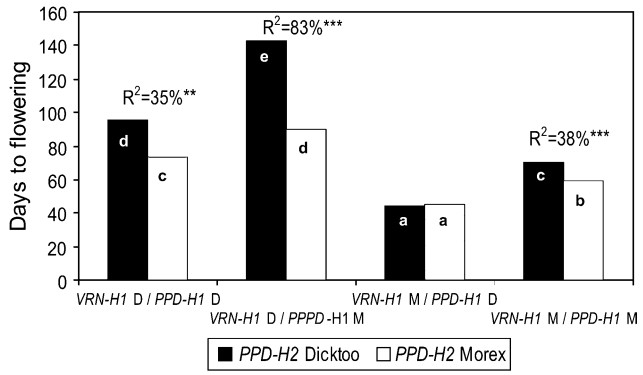

Fig. 2. Effects of three-way locus interactions between *VRN-H1*, *PPD-H1*, and *PPD-H2* on flowering time under synchronous photo and thermo cycles in the Dicktoo (facultative)×Morex (spring) barley population. Group averages with the same letter are not significantly different from each other at the P=0.05 level.

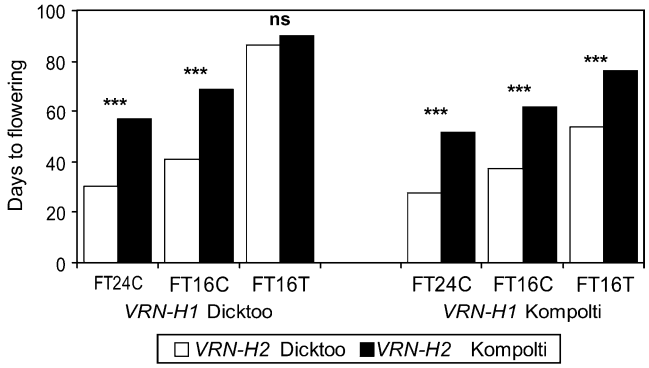

Fig. 3. Effects of daily fluctuating environmental factors on the association between the allele phases of *VRN-H2* and *VRN-H1* genes in the Dicktoo (facultative)×Kompolti korai (winter) barley mapping population.

flowering, with one exception: under 16T, the D allele at the *VRN-H1* locus resulted in extremely delayed flowering, irrespective of the allele phase at *VRN-H2*.

The *VRN-H3* locus had no significant effect on flowering time in the DK population, either alone, or in association with the allele phases of *VRN-H2* and *VRN-H1*.

Effects of Dicktoo alleles at the major flowering loci

The unique effects of D alleles at the flowering time loci monitored in these experiments show excellent correspondence across genetic backgrounds and growth conditions. For example, the flowering times of the subsets of lines with D alleles at all five *VRN* and *PPD* loci (6 lines in the case of DM and 10 lines in the case of DK populations) were not significantly different at 16C and 16T, in the two conditions which were identical for the two populations. The corresponding FT16C and FT16T means and *P* values for the 6 DM and the 10 DK lines were 40 d versus 42 d (P=0.33) and 99 versus 92 d (P=0.44), respectively.

Discussion

The primary environmental factors controlling the major developmental loci in cereals are low temperature on *VRN* loci (Yan *et al*., 2003, 2004, 2006) and photoperiod duration on *PPD* loci (Laurie *et al*., 2004; Turner *et al*., 2005; Beales *et al*., 2007). The associations between these environmental cues and corresponding genes were identified via QTL analyses and quantitative genetics approaches (Laurie *et al*., 1994, 1995; Karsai *et al*., 2005; Kóti *et al*., 2006; Szűcs *et al*., 2007) and by monitoring changes in gene activities (von Zitzewitz *et al*., 2005; Turner *et al*., 2005; Dubcovsky *et al*., 2006; Trevaskis *et al*., 2006; Beales *et al*., 2007). There is evidence, however, that secondary and interaction effects may also regulate these loci, i.e. photoperiod on *VRN* loci (von Zitzewitz *et al*., 2005; Dubcovsky *et al*., 2006; Karsai *et al*., 2006; Szűcs *et al*., 2006; Trevaskis *et al*., 2006).

Our results are novel in demonstrating the large effects of minor variations in environmental signals on flowering time. A 2 °C thermo cycle caused a delay in flowering time of 70 d as compared to a constant temperature. The phenotypic reactions of the two mapping populations were in accordance with the results observed for the parental lines in previous experiments (Karsai *et al*., 2008). Flowering of both populations was the earliest under constant environmental conditions and was the most delayed when long photoperiod cycles and thermo cycles of 2 °C were applied in synchronous fashion. It is shown that this variation in timing is due to allelic variation at four flowering time-related loci: *VRN-H1*, *VRN-H2*, *PPD-H1*, and *PPD-H2*, and certain inter-locus allelic interactions.

The application of synchronous photo and thermo cycles significantly delayed the flowering time of plants with Dicktoo type *VRN-H1* in both populations. The vernalization-critical regulatory regions of Dicktoo and Morex alleles at *BM5A*, the candidate gene at the *VRN-H1* locus, are polymorphic (Fu *et al*., 2005; von Zitzewitz *et al*., 2005), but identical between Dicktoo and Kompolti korai (Szűcs *et al*., 2007). The significant differences found between the effects of *VRN-H1* alleles on the flowering times of the two latter varieties may be due to as yet uncharacterized functional polymorphisms in other regions of the 17 kb gene or to the effects of a tightly linked gene, for example, *HvPhyC* (Szűcs *et al*., 2006). Support for the former possibility is that *BM5A* has been shown to respond to other environmental signals in addition to temperature: there is no transcription of the winter growth habit type Dicktoo *BM5A* allele under short photoperiod (Danyluk *et al*., 2003; von Zitzewitz *et al*., 2005). Further gene expression studies under the specific environmental conditions of fluctuating factors are necessary for determining which gene(s) may be responsible for the significantly delayed flowering associated with the Dicktoo *VRN-H1* locus. In addition to influencing the significance of alleles at the *VRN-H1* locus, the synchronously applied photo and thermo cycles also modified the interactions between *VRN-H1* and the other flowering time loci in both populations. In the Dicktoo×Morex population, environment-specific interactions between *VRN-H1*, *PPD-H1*, and *PPD-H2* loci could be detected.

HvPRR7, the candidate gene for the *PPD-H1* locus, has an *Arabidopsis* homologue (*AtPRR7*) that is directly involved in the central oscillator cycles of the circadian clock (Salomé and McClung, 2005; Turner *et al*., 2005). The activity of *HvPRR7* shows daily fluctuation, suggesting a connection with circadian rhythm in barley. A single nucleotide change in the CCT domain of *HvPRR7* results in a loss of function mutation of tremendous adaptive importance: mutant genotypes are insensitive to long photoperiods, leading to later flowering under higher latitude summer conditions. This mutation does not affect the expression of *HvPRR7* and its daily fluctuations in activity (Turner *et al*., 2005). Our results indicate that the effects of the synchronous photo and thermo cycling on the *PPD-H1* locus were independent of allele phase in the CCT domain (Fig. 1). Therefore, the observed phenotypic variation must be due to polymorphisms in other regions of the gene besides the CCT domain, or to another linked, but unknown, gene. Support for the former comes from the report that, in wheat, a large deletion in the promoter region of *PPD-D1* is responsible for the shift in peak activity within the daily circadian rhythm, resulting in altered circadian periodicity and, consequently, photoperiod insensitivity (Beales *et al*., 2007).

The epistatic interactions between *VRN-H1* and *PPD-H1* were previously identified in the Dicktoo×Morex population under long photoperiod treatments, both in greenhouse and in growth chamber tests (Pan *et al*., 1994; Karsai *et al*.,

1997). In the current experiments, it is confirmed that the combination of the recessive *BM5A* allele at *VRN-H1* and the insensitive *HvPRR7* allele at *PPD-H1* resulted in the latest flowering genotypes in each treatment. In plants with this allele combination, the allele phase at *PPD-H2* became a significant determinant of flowering time under synchronous photo and thermo cycles.

Allelic variation at the *PPD-H2* locus is of great agronomic importance: at the QTL level, this locus has significant effects on flowering time under short photoperiod conditions and in autumn field-sown experiments (Pan *et al*., 1994; Laurie *et al*., 1995; Cuesta-Marcos *et al*., 2008). *HvFT3* is the candidate gene for the *PPD-H2* locus (Faure *et al*., 2007). Under short-day conditions, the presence of this gene (characteristic of most spring barleys) is associated with early flowering, while the deletion of the gene (characteristic of most winter barleys) is associated with late flowering. The association between *HvFT3* gene and the *PPD-H2* locus is based on phenotype and allele associations, and gene expression patterns: *HvFT3* is expressed only under short photoperiods and is not detectable under long light regimes (Faure *et al*., 2007). Our results show that the *PPD-H2* locus is a significant determinant of flowering time under long photoperiods as well, but only in the case of applying synchronous photo and thermo cycles, and when specific allelic configurations are present at the *PPD-H1* and *VRN-H1* loci. The difference between our results and those of Faure *et al*. (2007) may be due to their use of a genotype with the 'early' (sensitive) allele at *PPD-H1* and a spring allele at *VRN-H1*. The largest delay of flowering due to the allele phase of the *PPD-H2* locus was observed in plants with the late flowering (insensitive) allele at *PPD-H1* and the repressible winter allele at *VRN-H1*. In this case, the null/deleted (sensitive) *HvFT3* allele at *PPD-H2* resulted in complete repression of flowering under a long photoperiod with synchronous photo and thermo cycles, while the presence of the *HvFT3* gene in *PPD-H2* resulted in significantly earlier flowering.

In the Dicktoo×Kompolti korai population, the epistatic interactions between *VRN-H2* and *VRN-H1* loci could be examined in detail. *ZCCT-H*, the candidate gene at the *VRN-H2* locus, is hypothesized to encode the transcriptional repressor of the winter allele at *VRN-H1* (Yan *et al*., 2004; Dubcovsky *et al*., 2006; Kóti *et al*., 2006; Szűcs *et al*., 2007). This gene is present in Kompolti korai and absent in Dicktoo. It was identified in the DK population as the only significant QTL for vernalization response (Karsai *et al*., 2005). *ZCCT* expression is highest prior to vernalization and decreases rapidly during vernalization (Yan *et al*., 2004). Photoperiod duration also affects *ZCCT* activity: a level of expression occurs under long, but not short photoperiods (Dubcovsky *et al*., 2006; Trevaskis *et al*., 2006). The connection between photoperiod sensitivity and the allele phase of the *VRN-H2* locus was also identified via functional QTL analyses (Karsai *et al*., 2006). Trevaskis *et al*. (2006) demonstrated that *ZCCT-H* gene expression levels fluctuate on a daily basis, suggesting that this gene is under circadian clock control. Under a long photoperiod and constant temperature it was found that the presence of *ZCCT-H* delayed flowering to the same extent (20–30 d) in plants with either the Dicktoo or the Kompolti *VRN-H1* alleles (Fig. 2). The application of synchronous photo and thermo cycles dramatically changed the epistatic interaction between *VRN-H2* and *VRN-H1*. Plants with Dicktoo type *VRN-H1* flowered late, independent of the presence/absence of the *ZCCT-H* gene (Fig. 2).

Fluctuations in light and temperature have more modest delaying effects on flowering time of barley under greenhouse conditions, where there are gradual and small changes in light and temperature (Szűcs *et al*., 2007), than under the conditions reported here. The synchrony, as well as magnitude, of cycling can be important: asynchronous cycling of temperature and photoperiod was reported to accelerate flowering in *Sorghum*, as compared to synchronous cycling (Morgan *et al*., 1987; Ellis *et al*., 1997). Morgan *et al*. (1987) suggested that the daily temperature cycles and the asynchrony between photo and thermo cycles could be an additional environmental signal to supplement photoperiodic control of development, and thus play an important role in entraining circadian rhythms.

In summary, the application of synchronous photo and thermo cycles under controlled environment conditions led to dramatic changes in the flowering time phenotype. A genetical dissection of these changes via QTL analysis revealed novel effects and interactions of barley *VRN* and *PPD* loci. It was found that the facultative growth habit and short-day sensitivity of Dicktoo is due to the *VRN-H2* deletion coupled with the recessive allele at *VRN-H1*, as previously described (von Zitzewitz *et al*., 2005), as well as the null (late-flowering) allele at the *PPD-H2* locus. This particular allele combination might render Dicktoo sensitive to fluctuations in photoperiod and temperature. We hypothesize that this cycling in photoperiod and temperature led to changes in the entrainment of the circadian rhythm, which, in turn, altered activity of *PPD-H1* and *VRN-H1*. Our results suggest that the *VRN-H2* and *PPD-H2* loci are also involved in the regulation of and/or are regulated by the circadian rhythm.

Supplementary data

Supplementary data can be found at *JXB* online.

Fig. S1. Phenotypic frequency distributions of flowering time in the Dicktoo×Morex (a–c) and Dicktoo×Kompolti korai (d–f) barley mapping populations under various combinations of daily fluctuating factors; (a, d) show continuous light and a constant temperature of 18 °C;

(b, e) show a 16 h photo cycle and a constant temperature of 18 °C; (c, f) show a 16 h photo cycle and a 18/16 °C thermo cycle applied synchronously.

Fig. S2. QTL analysis results of flowering time in two barley mapping populations under the three environmental conditions (24C, continuous light and constant temperature of 18 °C; 16C, 16 h photo cycle and constant temperature of 18 °C; 16T, 16 h photo cycle and 18/16 °C thermo cycle applied synchronously).

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