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Functional characterisation of *HvCO1*, the barley (*Hordeum vulgare*) flowering time ortholog of *CONSTANS*

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SUMMARY

Variation in photoperiod response is a major factor determining plant development and the agronomic performance of crops. The genetic control of photoperiodic flowering has been elucidated in the model plant Arabidopsis, and many of the identified genes are structurally conserved in the grasses. In this study, *HvCO1*, the closest barley ortholog of the key photoperiod response gene *CONSTANS* in Arabidopsis, was over-expressed in the spring barley Golden Promise. Over-expression of *HvCO1* accelerated time to flowering in long- and short-day conditions and caused up-regulation of *HvFT1* mRNA under long-day conditions. However, the transgenic plants retained a response to photoperiod, suggesting the presence of photoperiod response factors acting downstream of *HvCO1* transcription. Analysis of a population segregating for *HvCO1* over-expression and natural genetic variation at *Ppd-H1* demonstrated that *Ppd-H1* acts downstream of *HvCO1* transcription on *HvFT1* expression and flowering. Furthermore, variation at *Ppd-H1* did not affect diurnal expression of *HvCO1* or *HvCO2*. Over-expression of *HvCO1* increased transcription of the spring allele of *Vrn-H1* in long- and short-day conditions, while genetic variation at *Ppd-H1* did not affect *Vrn-H1* expression. Over-expression of *HvCO1* and natural genetic variation at *Ppd-H1* accelerated inflorescence development and stem elongation. Thus, *HvCO1* probably induces flowering by activating *HvFT1* whilst *Ppd-H1* regulates *HvFT1* independently of *HvCO1* mRNA, and all three genes also appear to have a strong effect in promoting inflorescence development.

Keywords: *Hordeum vulgare*, photoperiod, flowering, meristem, *HvCO1*, *Ppd-H1*.

INTRODUCTION

The time of flowering is crucial for the adaptation of plants to a given environment and has a major impact on grain yield in crop species (Cockram *et al.*, 2007a). Seasonal changes in photoperiod are major cues controlling development in many plant species. The photoperiod flowering pathway channels inputs from light, day length and the circadian clock to promote the floral transition. In Arabidopsis, CONSTANS (CO) is a central regulator of this pathway, triggering the transcription of the gene encoding the mobile florigen hormone FLOWERING LOCUS T (FT). The FT protein moves from the leaves through the phloem to the shoot apical meristem where it induces the switch from vegetative to reproductive growth (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007; Mathieu *et al.*, 2007; Jaeger and Wigge, 2007). *CO* is regulated at the transcriptional level by several genes that are part of the circadian clock or are under circadian clock control, so that *CO* mRNA accumulates at the end of a long summer day. At the protein level CO is regulated by photoreceptors and the ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) that respectively stabilise CO in light or de-stabilize CO in the dark (Jang *et al.*, 2008). As *CO* transcription occurs before dusk on long days (LD) but after dusk on short days (SD), CO protein only accumulates and mediates transcription of *FT* under LD (Turck *et al.*, 2008).

Orthologous genes of *CO* have been identified in many species, suggesting conservation of the components of the Arabidopsis photoperiod pathway. In both monocot and dicot species, CO activates *FT* or related genes under inductive day lengths to promote flowering (Suárez-López *et al.*, 2001; Hayama *et al.*, 2003). *Heading date 1* (*Hd1*), the rice ortholog of *CO*, promotes heading under SD conditions through the induction of *Hd3a*, which encodes a protein closely related to Arabidopsis FT (Yano *et al.*, 2000; Kojima *et al.*, 2002; Hayama *et al.*, 2003). Griffiths *et al.* (2003)

identified nine *CO* orthologs in barley (*Hordeum vulgare*), of which *HvCO1* shows the closest orthology to *CO* and *Hd1* based on the conserved CCT (CO, CO-like, TOC1) domain near the carboxy terminus and the two zinc finger B-boxes near the amino terminus. However, barley was previously shown to differ from rice in having two paralogous *CO*-like genes (*HvCO1* on chromosome 7H and *HvCO2* on chromosome 6H), of which only *HvCO1* is collinear with the rice *CO* ortholog *Hd1* (Higgins *et al*., 2010).

The role of *CO* orthologs in regulating flowering time in temperate cereals has not been elucidated, as no genetic variants in these genes or transgenic plants affecting their expression have been described. Griffiths *et al.* (2003) found that none of the *CO* orthologs in barley coincided with known flowering time quantitative trait loci (QTLs). On the other hand, Turner *et al.* (2005) suggested that variation in the major barley photoperiod response gene *Ppd-H1* affected flowering time through shifting the diurnal expression peaks of *HvCO1* and *HvCO2* mRNA into the dark phase. *Ppd-H1* is orthologous to the Arabidopsis clock gene *PRR7*, and a recessive mutation in the CCT domain of *Ppd-H1* causes photoperiod insensitivity and late flowering in spring barley (Turner *et al*., 2005). Wild and cultivated winter barley genotypes in contrast, carry the photoperiod responsive *Ppd-H1* allele, which induces early flowering in long photoperiods. Winter and spring barley genotypes are also distinguished by allelic variation at *Vrn-H1* and *Vrn-H2*, where deletions of the entire *Vrn-H2* gene and in the first intron of *Vrn-H1* cause vernalisation insensitivity in spring barley (Yan *et al*., 2004; Hemming *et al*., 2008).

In temperate cereals, the photoperiod and vernalisation pathways are closely intertwined. In wheat, the TaFT protein regulates transcription of the vernalisation-responsive MADS box transcription factor *VRN1* in the leaf (Li and Dubcovsky, 2008). Expression of *VRN1* and *TaFT* is necessary to induce flowering under LD conditions (Shimada *et al*., 2009). In addition, Kane *et al.* (2007) have proposed that *VRN1* expression in wheat is also controlled by the floral repressor TaVRT2, which is another MADS box transcription factor and the cereal ortholog of SHORT VEGETATIVE PHASE (SVP) in Arabidopsis (Hartmann *et al*., 2000). VRT2 was shown to bind to the CArG-box in the *VRN1* promoter and was proposed as a vernalisation-regulated repressor of *VRN1* (Kane *et al*., 2005, 2007). However, Trevaskis *et al.* (2007) have shown that SVP orthologs *HvVRT2*, *HvBM1* and *HvBM10* did not affect *Vrn-H1* expression levels and primarily delayed development after floral transition. These examples, suggest a close connection between the photoperiod and vernalisation pathways; however, the genetic interactions between photoperiod response and vernalisation genes are not yet clear.

In the present study, we functionally characterise *HvCO1* as the closest barley *CO* ortholog and its interaction with genetic variation at *Ppd-H1* and *Vrn-H1*, to further unravel the photoperiod response pathway in barley. We demonstrate that over-expression of *HvCO1* induces *HvFT1* expression and accelerates time to flowering. In addition, our data suggest that variation at *Ppd-H1* acts downstream of *HvCO1* mRNA on expression of *HvFT1* and flowering. Over-expression of *HvCO1* also up-regulated the spring allele of *Vrn-H1*, which suggested a photoperiod control of this vernalisation-responsive gene. Out data thus demonstrate that *HvCO1* functions in the control of flowering time in barley; however, the strong effect of *Ppd-H1* and control of *Vrn-H1* expression indicate modifications of the photoperiod response pathway in barley as compared to Arabidopsis.

RESULTS

Over-expression of *HvCO1* promotes flowering in barley but not in Arabidopsis

We tested whether *HvCO1* promotes flowering in barley by over-expressing *HvCO1* under the control of the maize ubiquitin promoter in the spring barley Golden Promise. Golden Promise carries the mutated, photoperiod-insensitive *ppd-H1* allele and the spring alleles at *Vrn-H1* and *vrn-H2*. Ten independent transgenic lines (4–12 plants per line) in T2 were tested for flowering time measured as days to heading under LD and SD photoperiods (Figure 1). Transgenic lines flowered on average 42 days after sowing and non-transgenic controls as well as the Golden Promise progenitor 62 days after sowing under LD conditions. Under SD conditions, transgenic lines flowered on average after 85 days, whereas non-transgenic control as well as Golden Promise plants had not flowered by 130 days after sowing, when the experiment was stopped. Over-expression of

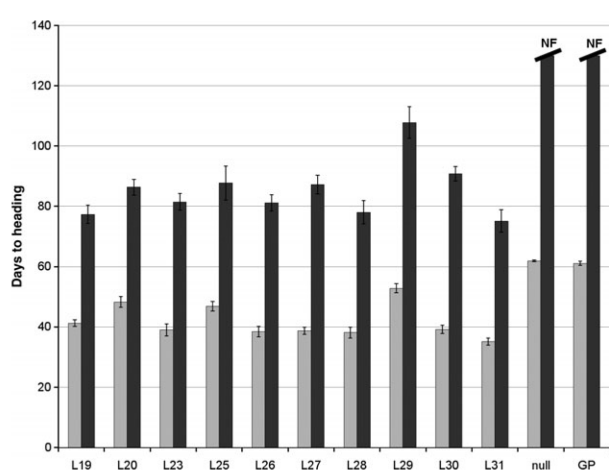

Figure 1. Flowering time of *Ubi::HVCO1* lines under long-day (LD) and short-day (SD) conditions.
Ten independent *Ubi::HVCO1* lines (L19 to L 31), the null segregant control (null) and the wild type (GP, Golden Promise) were grown under LD (grey bars; 16-h light) and SD (black bars; 8-h light). Flowering time is measured in days to heading; the null segregants and the wild type under SD did not flower until the experiment was stopped (NF, 130 days). Bars represent the average of 4–10 plants ± standard error.

HvCO1 thus promoted flowering under LD and SD conditions, but flowering time under SD was significantly delayed compared to LD.

To test whether *HvCO1* also promotes flowering in *Arabidopsis thaliana,* we expressed *HvCO1* under the CaMV35S promoter in the Arabidopsis *co-2* mutant. Two independent transgenic families carrying *35S::HvCO1*, the wild-type accession Landsberg *erecta* (L*er*), *35S::HA-CO co-2* and the Arabidopsis *co-2* mutant were scored for the number of leaves at bolting under LD and SD (10–12 plants per genotype/condition). The transgenic lines over-expressing *HvCO1* did not differ in development from the Arabidopsis *co-2* mutant, while Arabidopsis lines over-expressing *CO* in the *co-2* mutant background were early flowering (Figure S1a in Supporting Information). Reverse transcriptase-PCR analysis confirmed that the mRNAs of *HvCO1* and *CO* were over-expressed, but only over-expression of *CO* induced over-expression of *FT* in Arabidopsis (Figure S1b–d). Therefore, over-expressing *HvCO1*, a *CO*-like gene predicted to contain only one functional B-box (Griffiths *et al.*, 2003), did not promote *FT* expression and flowering in Arabidopsis.

Candidate gene expression in independent *Ubi::HvCO1* plants

Gene expression of candidate genes was analysed in the independent *Ubi::HvCO1* lines at the end of the day under LD and SD, when *HvCO1* and *HvFT1* mRNAs peak in expression. Expression analysis confirmed that *HvCO1* was over-expressed under LD and SD. *HvCO1* mRNA was three to ten times more abundant in transgenic plants compared with the non-transgenic controls (Figure 2a). Over-expression of *HvCO1* in the transgenic lines did show significant differences between LD and SD; however, these only explained 8% of the overall expression variance (Table S1). *HvFT1* was up-regulated at least five-fold in the transgenic lines under LD. Under SD, *HvFT1* mRNA levels were also up-regulated in two lines but to a much smaller extent than under LD (Figure 2c).

In addition, *Vrn-H1* was significantly up-regulated in transgenic lines compared with the non-transgenic control and Golden Promise under both LD and SD. However, there was a large variation in expression between transgenic lines. Transgenic line 30, for example, exhibited expression levels lower than the non-transgenic control (Figure 2d). *HvVRT2* was significantly down-regulated in the transgenic lines as compared with the null segregants and Golden Promise under LD, but not under SD (Figure 2e). *HvBM1* expression was significantly lower in the wild type and null segregants than transgenics under LD, but not under SD (Figure 2f). *HvCO2* and *HvBM10* expression levels were not significantly different between transgenic and wild-type lines and were characterized by a large variation in expression between transgenic lines (Figure 2b,g, Table S1). Expression of *HvFT2*, *HvFT3* and *HvFT4* was not detected in any genotype.

Over-expression of *HvCO1* and variation at *Ppd-H1* accelerate inflorescence development

The phenotypic effects of *HvCO1* over-expression on meristem development were scored according to the Waddington scale in two independent *Ubi::HvCO1* lines (19, 30) and Golden Promise. Plants were grown under SD for 21 days to allow for establishment of all transgenic plants, and then either transferred to LD or kept under SD. After transfer, replicate primary shoots were dissected every 2–3 days under LD and every 4–5 days under SD for all genotypes.

Transition to the reproductive stage as indicated by the formation of a 'double ridge' on the spike primordium (Waddington stage 2) occurred 8 days after and the beginning of stem elongation (Waddington stage 3) approximately 11 days after transfer to LD in transgenic and wild-type plants (Figures 3a and S2). However, stem elongation was significantly faster in transgenic lines than in Golden Promise. *Ubi::HvCO1* lines flowered 29 days after and Golden Promise more than 43 days after transfer to LD. Under SD conditions, the *Ubi::HvCO1* lines and Golden Promise showed similar patterns of meristem development as observed under LDs, so that genetic differences in meristem development were observed after the beginning of stem elongation (Waddington scale 3, Figure 3b).

Phenotypic effects of variation at *Ppd-H1* on meristem development were also analysed between the spring barley Scarlett (ppd-H1) and derived introgression lines (Ppd-H1). Like in *Ubi::HvCO1* lines, transition to a reproductive meristem occurred 8 days after and stem elongation (Waddington stage 3) began 11 days after transfer to LD in Scarlett and S42-IL107 (Figures 3c and S2). However, stem elongation and inflorescence development were accelerated in S42-IL107 as compared with Scarlett, flowering occurred after 29 days in S42-IL107 and after 43 days in Scarlett. In contrast to *Ubi::HvCO1* and Golden Promise, meristem development of Scarlett and S42IL-107 did not differ under SD, and neither line flowered under SD (Figure 3d). Over-expression of *HvCO1* and natural variation at *Ppd-H1* thus had a major effect on stem elongation and flowering under LD, and over-expression of *HvCO1* had similar effects under SD.

Over-expression of *HvCO1* and the active *Ppd-H1* allele up-regulate *HvFT1* and *Vrn-H1* expression

We tested the effects of *HvCO1* over-expression on the diurnal expression profiles of barley flowering time genes in leaf material of *Ubi::HvCO1* line 19 and Golden Promise under LD and SD. *HvCO1* was over-expressed in the *Ubi::HvCO1* line as compared with Golden Promise at all time points under LD and SD (Figure 4a). *HvCO2* expression was not significantly different between transgenic and wild-type barley lines under LD and SD (Figure 4b). *HvFT1* was up-regulated in the *Ubi*::HvCO1 line as compared with Golden Promise under LD, while *HvFT1* mRNA levels were

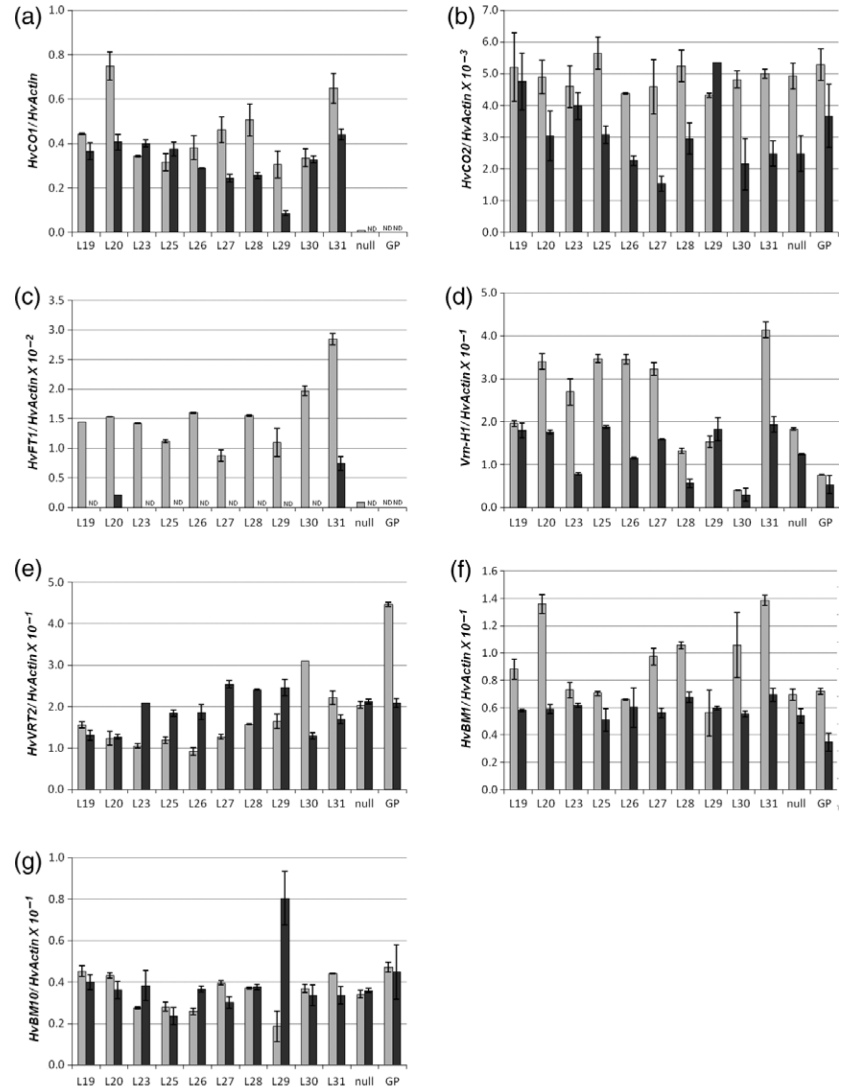

Figure 2. Expression of flowering time genes in *Ubi::HVCO1* lines under long-day (LD) and short-day (SD) conditions.
Ten independent *Ubi::HVCO1* lines (L19 to L31), the null segregant control (null) and the wild type (GP, Golden Promise) were grown under LD (grey bars; 16-h light) and SD (black bars; 8-h light). Bars represent the average expression of the target gene normalised to *HvActin* of two technical replicates ± standard error.

very low in both lines under SD (Figure 4c). *Vrn-H1* expression levels were higher in *Ubi*::*HvCO1* than Golden Promise under LD and SD (Figure 4d). *HvVRT2*, *HvBM1* and *HvBM10* exhibited a higher expression in Golden Promise than in *Ubi*::*HvCO1* (Figure 4e–g). Consequently, over-expression of *HvCO1* up-regulated *HvFT1* mRNA levels only under LD, while it increased *Vrn-H1* expression and down-regulated *HvVRT2*, *HvBM1* and *HvBM10* under LD and SD.

The effects of variation at *Ppd-H1* on diurnal expression patterns of flowering time genes were tested in Scarlett (*ppd-H1*) and S42-IL107 (*Ppd-H1*) under LD, as these lines only showed developmental differences under LD. The mutation in *Ppd-H1* did not affect the diurnal timing of *HvCO1* expression, but *HvCO1* mRNA levels were reduced in S42-IL107 compared with Scarlett with the mutated *ppd-H1* allele (Figure 5a). Similarly, diurnal expression patterns of *HvCO2* did not show clear diurnal differences between the two different *Ppd-H1* genotypes (Figure 5b). *HvFT1* and *Vrn-H1* mRNA levels were up-regulated in S42IL-107 compared with Scarlett (Figure 5c,d). *HvFT2* was expressed at low levels in S42IL-107, but not in Scarlett (data not shown). *HvVRT2*, *HvBM1* and *HvBM10* exhibited a higher expression in Scarlett than in S42IL-107 (Figure 5e–g). Consequently, substitution of the mutated *ppd-H1* allele by the photoperiod-sensitive *Ppd-H1* allele and over-expression of *HvCO1* had similar effects on downstream genes under LD, the up-regulation of *HvFT1* and *Vrn-H1* and down-regulation of *HvVRT2*, *HvBM1* and *HvBM10*. In addition, natural genetic variation at *Ppd-H1* did not shift diurnal expression peaks of *HvCO1* and *HvCO2* mRNAs.

Genetic analysis of flowering time in a population segregating for *Ubi::HvCO1*, *Ppd-H1* and *Vrn-H1*

Inheritance of phenotypic variation. We tested for interactions between *HvCO1* over-expression and variation at *Ppd-H1* and *Vrn-H1* in an F_2 population. This population was

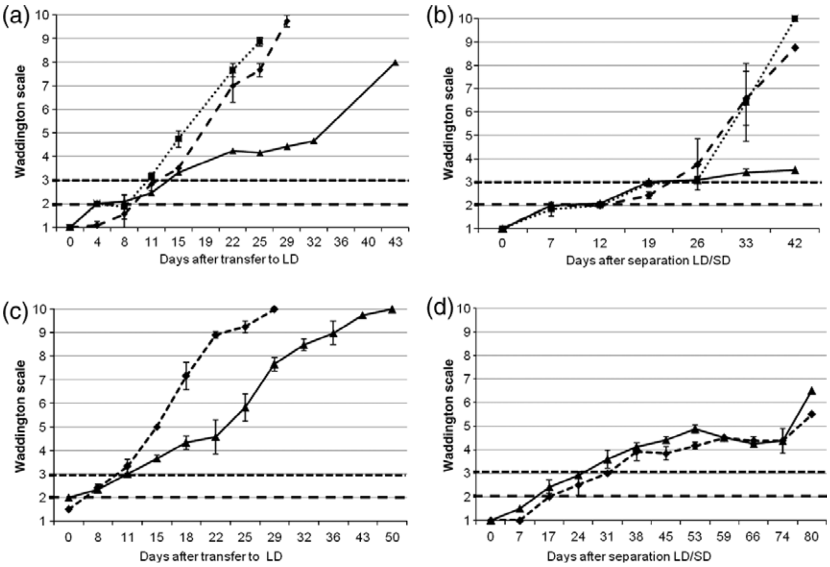

Figure 3. Effect of *HvCO1* over-expression and the *Ppd-H1* mutation on meristem development. Meristem development (Waddington scale) of two independent *Ubi::HVCO1* lines (dotted and dashed lines) and Golden Promise (solid line) under long days (LD; a, 16-h light) and short days (SD; b, 10-h light) and of the spring barley cultivar Scarlett (*ppd-H1*, solid line) and a derived near isogenic line S42IL-107 (*Ppd-H1*, dashed line) under LD (c) and SD (d). The dashed lines represent the transition to the reproductive meristem (Waddington scale 2) and the beginning of stem elongation (Waddington scale 3). Values represent the average of three plants ± standard error.

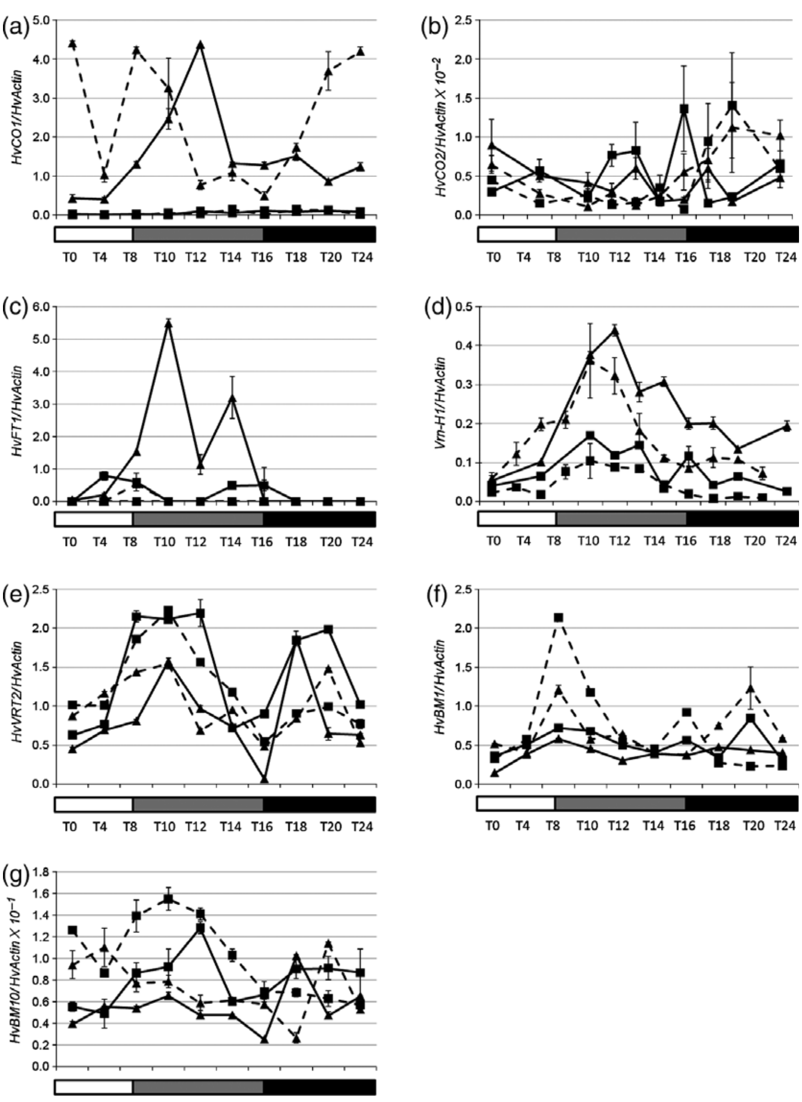

Figure 4. Diurnal expression of flowering time genes in *Ubi::HVCO1* lines under long-day (LD) and short-day (SD) conditions.

One *Ubi::HVCO1* lines (triangles) and the wild type (Golden Promise, squares) were grown under LD (solid lines; 16-h light) and SD (dashed lines; 8-h light). Transcript accumulation was measured at 2 to 4-h intervals by quantitative real-time RT-PCR analysis of specific genes and normalised to *HvActin*. Values represent the average of two technical replicates ± standard error.

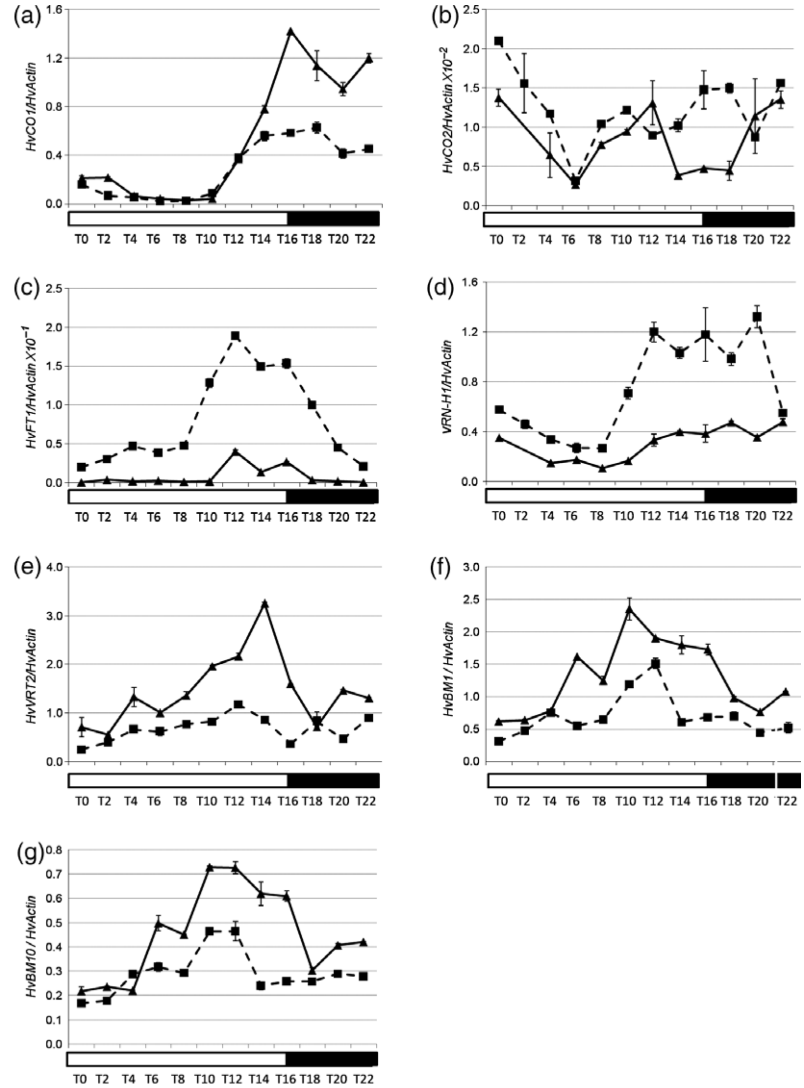

Figure 5. Diurnal expression of flowering time genes in the spring cultivar Scarlett (*ppd-H1*) and the introgression line S42IL-107 (*Ppd-H1*) under long days (LD).
Scarlett (solid line) and the derived near isogenic line S42IL-107 (dashed line) were grown under LD (16-h light). Transcript accumulation was measured at 2-h intervals by quantitative real-time RT-PCR analysis of specific genes and normalised to *HvActin*. Values represent the average of two technical replicates ± standard error.

generated by crossing *Ubi::HvCO1* in the background of Golden Promise with an introgression line derived from the winter barley Igri. This line carries the winter alleles at all flowering time loci, including *Ppd-H1* and *Vrn-H1*, but the spring allele at the *vrn-H2* locus as detected in the cross Triumph × Igri (Laurie *et al.*, 1995). An analysis of variance testing for the effects of the three known segregating genes under LD conditions, showed that variation at *Ppd-H1* explained most (32%) of the phenotypic variance in days to flowering observed in the population (Table 1). Presence or absence of the transgene only explained 21%, and genetic variation at *Vrn-H1* (spring/winter allele) 20% of the variation in flowering time. The interaction between *Ppd-H1* and *HvCO1* over-expression was significant, where the delay in flowering time in lines homozygous for the insensitive *ppd-H1* was counteracted by *HvCO1* over-expression (Figure 6a). The appearance of the first node on the stem which marks the beginning of stem elongation was affected by variation at *Vrn-H1* (45%) and at *Ppd-H1* (35%). F_2 lines with the sensitive *Ppd-H1* or spring *Vrn-H1* allele formed a first node significantly earlier than lines with the mutated *ppd-H1* or winter *vrn-H1* allele, while over-expression of *HvCO1* did not have a significant effect on the timing of this trait (Figure 6a,b). *Ppd-H1* and *Vrn-H1* had additive effects on flowering time, while variation at *Vrn-H1* only affected formation of the first node in the presence of a sensitive *Ppd-H1* allele (Figure 6c).

Under SD, flowering time was controlled by over-expression of *HvCO1,* but not by variation at *Ppd-H1* and *Vrn-H1.*

Inheritance of expression variation. Under LD, *HvFT1* expression was primarily controlled by *Ppd-H1* (30%), while genetic variation at *Vrn-H1* and the interaction between *Ppd-H1* and *Vrn-H1* explained 23 and 13% of the expression variance, respectively (Table 1). Over-expression of *HvCO1* also had a significant, but smaller (14%), effect on *HvFT1*

Table 1 ANOVA – association of days to heading (DH), appearance of the first node on the stem (Node) and gene expression of *HvCO1*, *HvFT1*, *Vrn-H1*, *HvVRT2*, *HvBM1* and *HvBM10* with genetic variation at *HvCO1* (*Ubi::HvCO1*), *Ppd-H1* and *Vrn-H1* and respective interactions under long-day (LD) and short-day (SD) conditions

	DH		Node		*HvCO1*		*HvFT1*		*Vrn-H1*		*HvVRT2*		*HvBM1*		*HvBM10*	
	F-stat	R^2	*F*-stat	R^2	*F*-stat	R^2	*F*-stat	R^2	*F*-stat	R^2	*F*-stat	R^2	*F*-stat	R^2	*F*-stat	R^2
LD																
HvCO1	43***	0.21	1	0.05	28***	0.57	21***	0.14	10**	0.13	9**	0.19	0.3	0.01	0.2	0.01
Ppd-H1	66***	0.32	7**	0.35	0	0.01	45***	0.30	1	0.01	5*	0.11	0.3	0.01	1.2	0.04
Vrn-H1	20***	0.20	4**	0.45	1	0.04	17***	0.23	21***	0.52	4*	0.18	0.8	0.07	1.3	0.08
HvCO1 × Ppd-H1	27***	0.13	1	0.04	3	0.06	4	0.02	1	0.02	3	0.06	1.1	0.05	0.5	0.02
HvCO1 × Vrn-H1	3	0.03	0	0.03	0	0.01	5	0.06	5**	0.11	1	0.04	1.2	0.10	3.8*	0.25
Ppd-H1 × Vrn-H1	3	0.03	1	0.14	0	0.01	10**	0.13	1	0.02	3	0.12	1.8	0.15	1.8	0.12
SD																
HvCO1	79***	0.87			41***	0.75	0.1	0.01	0	0.01	0	0.01	6.6*	0.28	1.1	0.07
Ppd-H1	0	0.00			1	0.02	0.1	0.01	0	0.01	1	0.03	3.2	0.14	0.4	0.03
Vrn-H1	1	0.01			1	0.02	0.9	0.15	3*	0.40	3	0.33	0.2	0.02	0.2	0.03
HvCO1 × Ppd-H1	0	0.00			1	0.02	0.0	0.00	0	0.01	0	0.00	1.0	0.04	0.1	0.01
HvCO1 × Vrn-H1	0	0.01			0	0.01	0.1	0.02	0	0.02	1	0.10	1.6	0.13	1.0	0.14
Ppd-H1 × Vrn-H1	1	0.02			0	0.00	0.4	0.07	0	0.02	0	0.01	0.2	0.01	0.9	0.12

F-stat, *F* statistics as calculated in the ANOVA; R^2, percentage of phenotypic variance explained by the respective factor.
Significant effects: **P* < 0.5; ***P* < 0.01; ****P* < 0.001.

expression. Expression of *HvFT1* revealed the highest correlations with flowering time and the beginning of stem elongation (appearance of first node) in the F_2 population (Table S2). *Vrn-H1* expression was mainly controlled by genetic variation at *Vrn-H1* (52%) and by over-expression of *HvCO1* under LD (13%). Interestingly, the interaction between *Vrn-H1* and the transgene was significant, where over-expression of *HvCO1*, but not variation at *Ppd-H1*, induced expression of the spring allele at *Vrn-H1* (Figure 7). The expression of *HvVRT2* was primarily controlled by over-expression of *HvCO1* (19%), but also by variation in *Ppd-H1* (11%) and *Vrn-H1* (18%). *HvVRT2* showed the highest negative correlation to *HvFT1* (–0.72) and *Vrn-H1* (–0.55) expression and was positively correlated with flowering time (0.48) and the beginning of stem elongation (0.67; Table S2). *HvBM1* and *HvBM10* expression was not significantly affected by *HvCO1* over-expression, or variation at *Ppd-H1* and *Vrn-H1*.

Under SD, over-expression of *HvCO1* had a significant effect on flowering time, and on expression of *HvCO1* and *HvBM1*. In addition, variation at *Vrn-H1* affected expression of *Vrn-H1*, but variation at *Ppd-H1* did not have any effect on gene expression under SD (Table 1).

DISCUSSION

HvCO1 over-expression causes early flowering in barley, but not in Arabidopsis

In *A. thaliana*, the CO protein comprises two B-Box-type zinc fingers proposed to be involved in protein–protein interactions (Khanna *et al.*, 2009). However, the *CO* orthologs, *Hd1* in rice and *HvCO* in barley, lack the highly conserved residues in the B-Box2 domain which are predicted to be required for B-box2 function (Griffiths *et al.*, 2003). Consistent with the requirement of B-box2 in *A. thaliana*, over-expression of *HvCO1* in the *co-2* mutant did not induce *FT* expression and rescue the late flowering phenotype in this study (Figure S1). In contrast, Martin *et al.* (2004) showed that constitutive expression of *LpCO*, the *CO* ortholog in *Lolium perenne*, also characterized by a non-conserved B-Box2, could complement the Arabidopsis *co-2* mutant. However, mutations in B-box2 of *HvCO1* correspond to those found in late-flowering *co* mutant alleles in Arabidopsis (Robson *et al.*, 2001) and our data suggest that two functional B-boxes are required for flower induction in Arabidopsis.

Onouchi *et al.* (2000) demonstrated that constitutive over-expression of *CO* from the CaMv35S promoter caused early flowering and almost complete insensitivity to day length in Arabidopsis. Similar to Arabidopsis, constitutive up-regulation of *HvCO1* in barley induced early flowering under LD and SD in the present study. However, flowering was delayed by on average 43 days in the transgenic lines grown under SD as compared to LD. Constitutive expression of *HvCO1* induced *HvFT1*, but the induction was significantly lower under SD as compared to LD and thus correlated with the differences in flowering between LD and SD (Figure 2c). These results suggest that photoperiod response factors in barley strongly control flowering time and *HvFT1* expression downstream of *HvCO1* mRNA.

Turner *et al.* (2005) suggested that *Ppd-H1* activates *HvFT1* expression by controlling the diurnal transcription of *HvCO1* and *HvCO2* and thus acts upstream of *HvCO1*. Over-expression of *HvCO1* and allelic substitution of *Ppd-H1* in a spring barley background both caused induction of

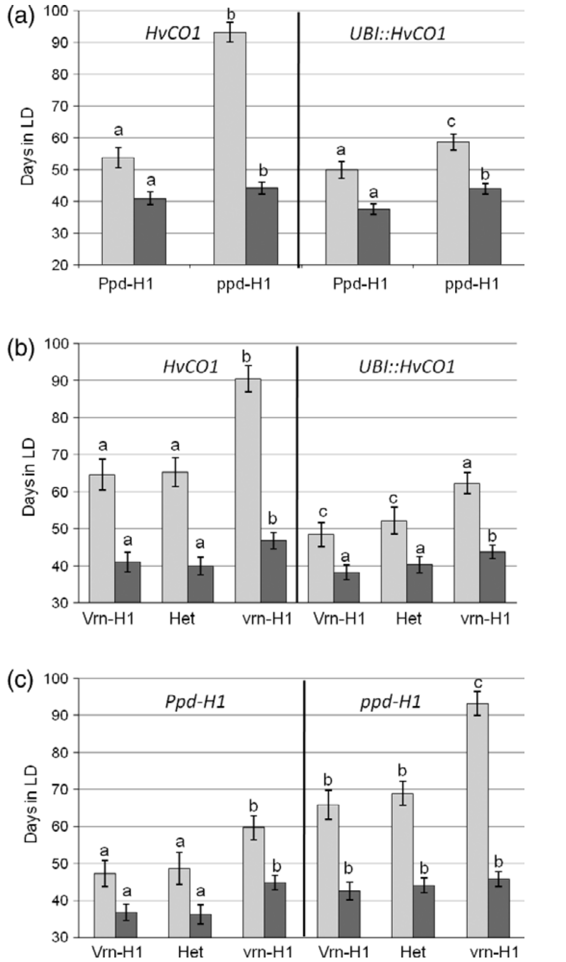

Figure 6. Interaction effects between *HvCO1* over-expression and natural genetic variation at *Ppd-H1* and *Vrn-H1* on flowering time (light grey bars) and stem elongation (dark grey bars).

The F_2 population *HvCO1*-ox × Igri (*vrn-H2*) derived by the cross between an introgression line in the background of the winter barley Igri (*Ppd-H1*, *vrn-H1*, *vrn-H2*) and two *Ubi::HVCO1* lines (*ppd-H1*, *Vrn-H1*, *vrn-H2*) was grown for 21 days under SD and then moved to LD and flowering time was recorded. The bars represent the average flowering time (grey bars) or time to appearance of first node (dark bars) for the different allelic combinations of *HvCO1*, *Ppd-H1* and *Vrn-H1* (±standard error). Letters indicate significant differences between phenotypic values of allelic classes.

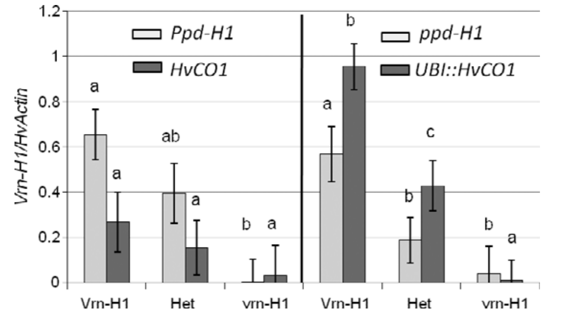

Figure 7. Effect of variation at *Ppd-H1* (light grey bars) and *HvCO1* over-expression (dark grey bars) on expression levels of winter and spring *Vrn-H1* alleles.

The F_2 population *HvCO1*-ox × Igri (*vrn-H2*) derived by the cross between an introgression line in the background of the winter barley Igri (*Ppd-H1*, *vrn-H1*, *vrn-H2*) and two *Ubi::HVCO1* lines (*ppd-H1*, *Vrn-H1*, *vrn-H2*) was grown for 21 days under short-day conditions (SD) and then moved to long days (LD). Bars represent *Vrn-H1* expression normalised to *HvActin* of the average number of lines having the indicated allelic combination ± standard error. Letters indicate significant differences between *Vrn-H1* expression of allelic classes separately for lines varying for *HvCO1* and *Ppd-H1*.

HvFT1 and *Vrn-H1* expression under LD (Figures 4c,d and 5c,d). However, variation at *Ppd-H1* did not affect diurnal expression patterns of *HvCO1* and *HvCO2* mRNAs (Figure 5a,b). Up-regulation of *HvFT1* by *HvCO1* over-expression and the dominant *Ppd-H1* allele demonstrated that both genes converge on *HvFT1*. These results suggested that *Ppd-H1* may act as a photoperiod response factor downstream of *HvCO1* transcription. The lower expression of *HvCO1* in the introgression line (*Ppd-H1*) than in Scarlett (*ppd-H1*) may reflect the more advanced developmental stage of the introgression line, and may not be a direct effect of variation at *Ppd-H1.*

Genetic variation at *Ppd-H1* acts downstream of *HvCO1* expression on *HvFT1*

In the present study, analysis of the barley F_2 population showed that variation at *Ppd-H1* orthologous to *PRR7* had a stronger effect on flowering time than over-expression of *HvCO1*. Variation at *Ppd-H1* as identified by Turner *et al.* (2005) thus affected flowering time in barley independent of *HvCO1* transcript levels and can explain the differences in flowering time between LD and SD observed in the transgenic lines (Figure 1). Differences in flowering time between LD and SD were also observed in the transgenic lines with the mutated *ppd-H1* allele from Golden Promise (Figure 1). Consequently, the mutated *ppd-H1* seems to be a hypomorphic allele with a reduced, but measurable, photoperiod response as compared with the wild-type dominant allele. The expression of *HvFT1* with the highest correlation to flowering time was primarily controlled by *Ppd-H1* (Table S2 and Table 1). Kikuchi *et al.* (2009) have already demonstrated that over-expression of *HvFT1* in rice induced flowering and concluded that *HvFT1* is the key gene responsible for flowering in the barley *FT*-like gene family (Faure *et al.*, 2007). Variation at *Ppd-H1* acted independently of *HvCO1* mRNA on *HvFT* expression, but could be a factor modifying the HvCO1 protein, for example by stabilizing or activating the protein. Valverde *et al.* (2004) and Jang *et al.* (2008) have shown in Arabidopsis that post-translational regulation of the CO protein is crucial for its response, by stabilizing the protein at the end of a LD, and promoting its degradation throughout the night under LD and SD. In addition, Ishikawa *et al.* (2011) showed that in rice PhyB mediated light-induced suppression of *Hd3a* through post-translational modification of the Hd1 protein. On the other hand, Fujiwara *et al.*

(2008) had already demonstrated for Arabidopsis that mutations in two other circadian clock genes *LHY* and *CCA1* delayed flowering and suppressed *FT* expression independent of the CO protein, but through the stabilization of the MADS box transcription factor *SVP*. Likewise, *Ppd-H1,* orthologous to the Arabidopsis clock gene *PRR7,* may also have affected *HvFT1* independently of *HvCO1* through the *SVP*-like genes *HvVRT2*, *HvBM1* or *HvBM10*. In the present study, barley lines carrying the transgene or the dominant *Ppd-H1* allele showed a lower level of *HvVRT2*, *HvBM1* and *HvBM10* expression; however, there was no clear difference between the *Ubi*::*HvCO1* and S42IL-107 lines, indicating that these genes were down-regulated during barley reproductive development rather than being directly affected by variation at *Ppd-H1* (Figures 4 and 5).

HvCO1* up-regulates the spring allele of *Vrn-H1

Flowering time and *HvFT1* expression in the F_2 population were also affected by the allelic status at *Vrn-H1* even in the absence of *Vrn-H2*. Trevaskis *et al.* (2006) and Casao *et al.* (2011) have shown that in winter barley, *Vrn-H1* only indirectly controls *HvFT* levels through the down-regulation of *Vrn-H2*, which is a repressor of *HvFT1*. However, in the present study, genetic variation at *Vrn-H1* explained 17% of the variance in *HvFT1* expression, where the winter allele at *Vrn-H1* delayed time to flowering and reduced *HvFT1* expression levels as compared to the spring allele (Table 1). Variation at *Vrn-H1* thus controlled *HvFT1* expression, suggesting that even in the absence of *Vrn-H2*, *Vrn-H1* directly or indirectly controls *HvFT1*.

Interestingly, *HvCO1* over-expression, but not variation at *Ppd-H1*, induced the expression of the spring *Vrn-H1* allele under LD (Figure 7). These results indicate that up-regulation of the spring *Vrn-H1* allele was directly induced by *HvCO1* over-expression and was not an indirect effect of the more advanced development of F_2-lines over-expressing *HvCO1*. Hemming *et al.* (2009) demonstrated that a region in the first intron of the *Vrn-H1* gene was required to maintain low levels of *Vrn-H1* expression prior to winter. Golden Promise carries a deletion of 5.2 kb in the first intron which is associated with increased *Vrn-H1* expression independent of vernalisation (Hemming *et al.*, 2009). Our results indicated that the photoperiod response pathway may be involved in the up-regulation of the spring allele of *Vrn-H1*, but fails to induce the winter allele prior to vernalisation. Kane *et al.* (2005, 2007) have suggested that VRT2 represses *VRN1* and that down-regulation of *VRT2* by cold allows expression of *VRN1*. In contrast, our results suggest that *HvVRT2* acts downstream of *Vrn-H1* expression. High correlations of *HvVRT2* expression with time of stem elongation and *HvFT1* expression indicated that *HvVRT2* down-regulation occurred during development and was not a direct effect of *HvCO1* over-expression. Trevaskis *et al.* (2007) have already shown that *SVP*-like genes including *HvVRT2* regulate meristem identity, but are unlikely to mediate de-repression of *Vrn-H1* during vernalisation. Interestingly, *TaVRT2* maps to the short arms of group 7 chromosomes in a region associated with QTLs involved in heading date in wheat and barley (Kane *et al.*, 2005; von Korff *et al.*, 2006, 2010; Wang *et al.*, 2009) and may thus play a role in the integration of photoperiod and vernalisation signals.

***HvCO1* over-expression and variation at *Ppd-H1* control late reproductive meristem development in barley**

Three major phases in cereal meristem development can be distinguished: (i) the vegetative phase until formation of the double ridge in the apex, (ii) spikelet growth until formation of stamen initials and (iii) spike growth until cessation of peduncle elongation (Slafer, 2003; Nicholls and May, 1963). It has been shown that the timing and duration of the different developmental phases vary independently and are determined genetically in response to the environment (Gonzalez *et al.*, 2005; Whitechurch *et al.*, 2007). Analyses of development in wheat grown under artificially manipulated photoperiods have already shown that the stem elongation phase was the most sensitive to changes in photoperiod (Slafer *et al.*, 2001). Accordingly, over-expression of *HvCO1* and natural variation at *Ppd-H1*, both genes implicated in the photoperiod control of flowering in barley, primarily affected stem elongation and late reproductive meristem development in the present study (Figure 3). Hemming *et al.* (2008) also showed that natural variation at *Ppd-H1* had little influence on the time to double ridge, but mainly accelerated development after the initiation of inflorescence in lines with active *VRN1* alleles grown in LD. In the present study, differences in stem elongation were affected by variation at *Ppd-H1* and *Vrn-H1*, and correlated with differences in the expression of *HvFT1*. *Vrn1* has already been proposed to be implicated in stem elongation in wheat (Chen *et al.*, 2009). Furthermore, studies in rice and tomato have also suggested that FT may have effects beyond floral induction on the development and architecture of the inflorescences (Lifschitz *et al.*, 2006). In rice, high levels of *Ghd7*, encoding a CCT domain protein, and reduced expression of *Hd3a* correlated with late flowering, but also with differences in panicle size, shape and seed number (Xue *et al.*, 2008). Similarly, heterozygosity of the loss-of-function allele of SINGLE FLOWER TRUSS (SFT), the tomato ortholog of FT, caused a shift in the balance of vegetative to reproductive growth and increased the total number of inflorescences and flowers per plant (Krieger *et al.*, 2010). Although the genetic pathways underlying vegetative phase change are now well understood in model plants (Amasino, 2010), the source and identity of signals that initiate the transition between the juvenile to adult stages are relatively unknown (Chuck *et al.*, 2007; Yang *et al.*, 2011). Flowering in barley occurs several weeks after formation of the double-ridge SAM morphology which marks the transition to the reproductive meristem.

These morphological changes may in fact indicate transition to an adult vegetative phase and reproductive competence which enables plant to respond to long photoperiods.

CONCLUSION

Functional analysis of *HvCO1* in a spring and a winter barley background allowed further characterisation of the flowering time pathway in barley. Earlier work had already demonstrated that despite structural conservation of flowering time genes across species, functional modifications are common. For example, vernalisation response is mediated by FLC in Arabidopsis, VRN1 in winter cereals (Trevaskis *et al.*, 2006) and BvFT1 in sugar beet (Pin *et al.*, 2010). In Arabidopsis, *CO* functions as a central gene in the photoperiodic induction of flowering by linking the circadian clock mechanism to the genes for meristem identity. In barley over-expression of *HvCO1* accelerated flowering; however, natural genetic variation in *Ppd-H1* controlled flowering independent of *HvCO1* mRNA. *Ppd-H1* may thus bypass the established *CO*-FT interaction in Arabidopsis to induce flowering under long days. In addition, the morphology of flower induction differs between cereals and Arabidopsis which may have necessitated the functional modifications of flowering time orthologs, illustrated by the prominent role of FT in the late reproductive development of barley versus its function in the transition to the reproductive meristem in Arabidopsis.

EXPERIMENTAL PROCEDURES

Plant material and cultivation

Transgenic 35S::HvCO1 Arabidopsis lines. The *constans-2* (*co-2*) mutant line in the L*er* background (Putterill *et al.*, 1995) was transformed with cDNA of *HvCO1* cloned by recombination using LR clonase II (Invitrogen, http://www.invitrogen.com/) into p35S::GATEWAY destination vectors under the control of the CaMV35S promoter (An *et al.*, 2004). In addition, the *co-2* mutant was transformed with 3× hemagglutinin (HA) tagged CO under the control of CaMV3*5S* promoter in the pGreen binary vector (GC and W. Soppe, Max Planck Institute for Plant Breeding Research, Cologne, Germany, unpublished data). The binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) or GV3101 (pMP90RK) and transformed into Arabidopsis L*er co-2* by the floral-dip method (Clough and Bent, 1998).

For flowering-time measurements in the co-2 mutant, L*er*, 35S::HA-CO co-2 and 35S::HvCO1 co-2 Arabidopsis lines, plants were sown on soil and grown in controlled environment chambers under LD (16-h light/8-h dark) or SD (8-h light/16-h dark) at a constant temperature of 21°C, and numbers of leaves at bolting were counted. Ten-day-old seedlings were harvested at zeitgeber time, T12, for RNA extraction and expression analysis. The SD samples were harvested in the dark.

Transgenic Ubi::HvCO1 lines. Golden Promise plants were transformed with an over-expression construct made by ligating cDNA clones of *HvCO1* (AF490468) to the maize ubiquitin promoter (Christensen *et al.*, 1992), and the resulting over-expression cassette was inserted into the pWBVEC8 binary vector (Wang *et al.*, 1998). Barley plants were transformed using *Agrobacterium* transformation of excised embryos (Tingay *et al.*, 1997; Matthews *et al.*, 2001). An average of 30 independent transformants were produced, and T1, T2 and T3 plants were screened for segregation of the transgene using primers that amplify the hygromycin selectable marker gene and the *HvCO1* cDNA sequence (Table S3). Ten independent transgenic T2-families designated *Ubi::HvCO1* lines, the null segregant controls for the above transgenic lines and the corresponding wild type Golden Promise were sown in soil and grown under LD (16-h light/8-h dark) and SD (8-h light/16-h dark) in the greenhouse (temperature 20°C/16°C days/nights), and flowering time (measured in days to heading) was scored. Leaf material from three plants for each T2 family was harvested 14 days after sowing at the end of the day (T14 under LD and T6 under SD) for RNA extraction and expression analysis. *Ubi::HvCO1* line 19 was additionally analysed for diurnal gene expression, and *Ubi::HvCO1* lines 19 and 30 for meristem development (see below).

F_2 population segregating for *Ubi::HvCO1, Ppd-H1* and *Vrn-H1*

Two randomly selected *Ubi::HvCO1* lines (lines number 19 and 30 in T3) were crossed with an introgression line derived from the winter barley Igri. Golden Promise carries the photoperiod-insensitive *ppd-H1* allele and spring alleles at *Vrn-H1* and *vrn-H2*. The introgression line is a BC4F2 selection from the DH population Igri × Triumph and carries the Igri alleles at all flowering time QTLs, the photoperiod-sensitive *Ppd-H1* allele, the winter allele at *vrn-H1,* but the spring allele *vrn-H2* from Triumph (Laurie *et al.*, 1995). The derived F_2 population was grown in soil in the greenhouse for 21 days under SD and then either transferred to LD or kept under SD (20°C/16°C days/nights). Plants were analysed for flowering time under LD (46 lines) and SD (42 lines) and leaf material was harvested to extract DNA for genotyping to test for the presence of the transgene and functional polymorphisms at *Ppd-H1* (Turner *et al.*, 2005) and *Vrn-H1* (Cockram *et al.*, 2007b; Table S3). In addition, leaf material was harvested from 25 F_2 lines 7 days after exposure to LD (T14) and from 20 F_2 lines under SD 14 days after separation of plants between LD and SD (T8) for RNA extraction and analysis of gene expression. F_2 lines were selected to allow for a balanced allelic representation at *HvCO1*, *Ppd-H1* and *Vrn-H1*.

Natural variation for *Ppd-H1/ppd-H1*

The German spring barley cultivar Scarlett and an introgression line S42IL-107 derived by crossing Scarlett with a wild barley accession ISR42-8 from Israel were used to test for the effects of natural variation at *Ppd-H1* on flowering and expression of putative downstream genes (von Korff *et al.*, 2004; Schmalenbach *et al.*, 2011). The spring cultivar Scarlett carries the insensitive *ppd-H1* allele and flowers late under long photoperiods, while the introgression line S42IL-107 carries the photoperiod-responsive *Ppd-H1* allele introgressed from wild barley and flowers early under long photoperiods. Scarlett and S42IL-107 were analysed for diurnal gene expression, meristem development and flowering time (see below).

Plant development and expression analysis

Meristem dissection and diurnal expression analysis. The two *Ubi::HvCO1* lines 19 and 30 (selected for crossing to the mutant winter barley line), Scarlett and S42IL-107 were grown for 21 days under SD and then either transferred to LD or kept under SD. After transfer, three replicate primary shoots per genotype were dissected every 2–3 days under LD and every 4–5 days under SD, and the development of the meristem was scored until flowering according to the Waddington scale (Waddington *et al.*, 1983). In addition, diurnal gene expression was tested in *Ubi::HvCO1* line 19 and Golden Promise under LD and SD, and in Scarlett and S42IL-107 only under LD as the latter did not show any developmental differences under SD. At the transition to the reproductive meristem (Waddington scale 2), leaf samples were harvested every 2 h for a total of 24 h starting in the morning at lights on (T0) under LD and SD simultaneously. For each sample, two pooled plants, and two biological replicates were collected. Samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

The F_2 lines under LD were scored for the formation of the first node on the main stem which marks the beginning of stem elongation (Chen *et al.*, 2009).

RNA extraction, cDNA synthesis and real time quantitative RT-PCR

Total RNA was extracted from 100 mg of tissue using TRIzol® reagent (Invitrogen) following the manufacturer's instructions, except for the use of RNaseH, followed by a DNase treatment (final volume 100 μl). First-strand cDNA synthesis was performed on 4 μl of total RNA using 100 U of SuperScriptTM II RT (Invitrogen) and 500 ng of poly-T primer and following the manufacturer's recommendations (final volume 40 μl). All samples within an experiment were reverse transcribed at the same time and the resulting cDNA was diluted 1:4 in nuclease-free water and stored in aliquots at −20°C.

Real-time quantitative (q) RT-PCRs were performed on cDNA samples using gene-specific primers (Table S3). Amplifications were performed using 1 μl of cDNA, 1 U of GoTaq Flexi DNA polymerase (Promega, http://www.promega.com/), 0.2 mM deoxynucleotide triphosphate (dNTP), 2.5 mM $MgCl_2$, 0.2 μM of each primer and 1 μl of EvaGreen (Biotium, http://www.biotium.com/). Reactions were performed on a LightCycler480 (Roche, http://www.roche.com/) with the following amplification conditions: 95°C for 5 min, 40 cycles of 95°C (10 sec), 60°C (10 sec) and 72°C (10 sec). Appropriate non-template controls were included in each 384-well PCR reaction, and dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. Starting amounts for each data point were calculated based on the titration curve for each target gene and the reference (*HvActin*) gene using the LightCycler 480 Software (Roche; version 1.5).

Statistical analysis

Significant differences in flowering time and gene expression between *Ubi::HvCO1* lines and wild type grown under LD and SD were calculated using a mixed model analysis of variance (ANOVA). The response (Y) was modeled according to the following three-factorial layout (SAS version 9.1; SAS Institute 2009):

$$Y_{ijkm} = \mu + P_k + G_i + T_j(G_i) + P_k \times G_i + E_{ijkm},$$

where μ is the general mean, P_k is the fixed effect of the *kth* treatment (LD or SD), G_i is the fixed effect of the i-th genotype (*Ubi::HvCO1* or WT), T_j is the random effect of the j-th transformant within the i-th genotype. The remaining terms describe the first order interaction effect. E_{ijkm} is the error of Y_{ijkm}.

Significant effects of the transgene and variation at the candidate genes *Ppd-H1* and *Vrn-H1* on flowering time and gene expression in the F_2 population under LD and SD were calculated using a fixed model ANOVA and a three-factorial layout:

$$Y_{ijkm} = \mu + C_i + P_j + V_k + C_i \times P_j + C_i \times V_k + P_j \times V_k + E_{jikm},$$

where μ is the general mean and E_{ijkm} is a modified residual error term. C_i, P_j and V_k are the fixed effects for the genotype levels for *HvCO1* (*Ubi::HvCO1* or wild type), *Ppd-H1* (*Ppd-H1* or *ppd-H1*) and *Vrn-H1* (*Vrn-H1*, heterozygote, *vrn-H1*), respectively. The remaining terms describe first-order interaction effects. Three-way interactions were subsumed in the residual error, as for some allele combination just one line replicate (m = 1) was tested.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Flowering time of Arabidopsis *co-2* mutant lines complemented with barley *HvCO1* under long-day and short-day conditions.

Figure S2. Effects of *HvCO1* over-expression and variation at *Ppd-H1* on meristem development.

Table S1. ANOVA for flowering time and gene expression in 10 independent *Ubi::HvCO1* families and wild type grown under long-day and short-day conditions.

Table S2. Correlations (Pearson coefficients) of flowering time and gene expression in the F_2 population *Ubi::HvCO1* × Igri (*vrn-H2*).

Table S3. Primer sequences.

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