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Running Title: CONSTANS controls VERNALIZATION2 in barley

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Research Area: Genes, Development and Evolution

CONSTANS controls floral repression by upregulating *VERNALIZATION 2* (*VRN-H2*) in barley

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Summary: The functional characterization of *CONSTANS* homologs provides new insights into the control of floral repression before vernalization in barley.

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Abstract

In barley (*Hordeum vulgare*), *PHOTOPERIOD 1* (*Ppd-H1*) acts as a major positive regulator of flowering under long day conditions, while *VERNALIZATION2* (*VRN-H2*) is a strong repressor of flowering under long days before vernalization. By contrast, *CONSTANS* (*CO*) plays a key role in the photoperiodic regulation of flowering in *Arabidopsis thaliana*. Here, we study the role of the closest barley *CO* homologs *HvCO1* and *HvCO2* in the long day dependent control of flowering and their interactions with *Ppd-H1* and *VRN-H2*. *HvCO2* overexpression in spring barley, with a natural deletion of the *VRN-H2* locus, caused a *Ppd-H1* dependent induction of flowering and *FLOWERING LOCUS T1* (*HvFT1*) expression. In winter barley, which carries the *VRN-H*2 locus, overexpression of *HvCO1/CO2* caused an upregulation of *VRN-H2* resulting in a reduced expression of *HvFT1* and delayed flowering under long and short day conditions. In addition, natural variation at *Ppd-H1* altered the expression of *VRN-H2* in wild type plants under long days. *VRN-H2* in turn was involved in the downregulation of *Ppd-H1* and *HvCO2* demonstrating strong reciprocal interactions between *HvCO2*, *Ppd-H1* and *VRN-H2*. Consequently, this study showed that the induction of the floral repressor *VRN-H2* and floral activator *HvFT1* was regulated by the same genes, *Ppd-H1* and *HvCO1/CO2*. Our findings provide a novel insight into the photoperiodic regulation of the vernalization pathway in barley.

Introduction

Flowering is one of the most critical stages in the life cycle of plants. Coincidence of flowering with favorable conditions ensures that seed production is maximized and enhances the chances of successful reproduction. A key adaptive mechanism to achieve this coincidence is sensing changes in day length, or photoperiod (Greenup et al., 2009). Long photoperiods promote flowering in the model and facultative long-day (LD) plant *Arabidopsis thaliana* through the activity of *CONSTANS* (*CO*), a transcription factor that binds to the promotor of *FLOWERING LOCUS T* (*FT*) which in turn induces the floral transition (Putterill et al., 1995; Tiwari et al., 2010). *CO* encodes a protein with two zinc finger B-boxes and a CCT domain (*CONSTANS*, *CONSTANS*-like and *TIMING OF CAB EXPRESSION1*: *TOC1*, Robson et al., 2001). *CO* transcription is regulated by the circadian clock and its components in a way that allows the accumulation of *CO* mRNA at the end of the light period of long days but after dusk in short days (Imaizumi et al., 2005; Fornara et al., 2009). The CO protein is stabilized by photoreceptors in the light and degraded by the ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) during the dark, which allows the accumulation of CO at the end of a long day to induce *FT* transcription (Jang et al*.,* 2008; Turck et al., 2008).

The function of CO in controlling the photoperiod response is conserved in the short-day (SD) cereal monocot rice (*Oryza sativa*). Under inductive SDs, *Heading date 1* (*Hd1*), the rice ortholog of CO, promotes flowering by inducing the expression of *Hd3a*, the ortholog of *FT (*Kojima et al., 2002; Izawa et al., 2002). Under LDs, however, *Hd1* represses flowering through the downregulation of *Hd3a* (Yano et al., 2000, Izawa et al., 2002; Hayama et al., 2003). Consequently, *Hd1* is bifunctional in rice where it promotes heading under SD conditions and inhibits it under LD conditions (Yano et al., 2000). In barley (*Hordeum vulgare*), *HvCO1* and *HvCO2* are the closest homologs of Arabidopsis *CO* and rice *Hd1* (Griffiths et al., 2003). Comparison with wheat, Brachypodium and rice suggests that *HvCO1* and *HvCO2* are paralogs that have arisen in temperate cereals by segmental duplication. *HvCO1* is colinear with *Hd1,* whereas *HvCO2* was lost in rice (Higgins et al., 2010). Overexpression of *HvCO1* promoted flowering under LD and SD conditions, which suggested that *HvCO1* functions as a floral activator in barley (Campoli et al., 2012). However, the role of *HvCO2* in flowering time control in barley has not yet been elucidated.

Comparison of CO function across species demonstrates that *CO* homologs may act as a LD activator of flowering as seen in Arabidopsis or a LD repressor of flowering as observed in rice. Nemoto et al. (2003) reported that wheat *CO* complemented *hd1* and repressed flowering in rice under LDs, suggesting that functional differences of *CO* in SD and LD plants are not due to structural variation but rather due to trans-acting regulatory mechanisms.

In rice, LD repression of flowering is mediated by two additional CCT-domain genes; *Hd2/PSEUDO-RESPONSE REGULATOR 37* (*OsPRR37*) and *Hd4/GRAIN NUMBER*, *PLANT HEIGHT AND*

HEADING DATE 7 (*Ghd7;* Koo et al., 2013; Gao et al., 2014; Xue et al., 2008). *OsPRR37* is orthologous to the Arabidopsis circadian clock gene *PRR3*/7 and is characterized by a pseudo receiver and a CCT domain. *OsPRR37* is expressed under LD and SD conditions but is only functional to repress *Hd3a* under LDs (Murakami et al., 2003; Koo et al., 2013; Gao et al., 2014). Interestingly, *Ppd-H1*, the barley homolog of the LD repressor *OsPRR37*, is the major photoperiod response gene in barley and induces flowering under LDs by upregulating *HvFT1*, the barley homolog of *Hd3a* (Turner et al., 2005). Barley carries five *FT* homologs of which *HvFT1* correlates with flowering time under long day conditions, while a natural deletion at *HvFT3* has been associated with floral development under short-day conditions (Yan et al., 2006; Faure et al., 2007; Kikuchi et al., 2009). *FT1* expression and flowering time are controlled by *PPD1* independently of *CO1/2* expression in barley and wheat (Wilhelm et al., 2009; Shaw et al., 2012; Campoli et al., 2012). Consequently, *CO* and *PRR37* may act independently and function as floral repressors or activators depending on the species and photoperiod. The genetic basis of this dual role of *CO* and *PRR37* as activators and repressors of flowering is not yet understood.

Ghd7 belongs to the CMF (CCT MOTIF FAMILY) subclass of the CCT gene family with only a single CCT domain (Cockram et al., 2012). *Ghd7* is upregulated under LD conditions and represses *Hd3a* and flowering time. *VERNALIZATION2 (VRN-H2*), a barley homolog of the LD repressor *Ghd7* in rice, is also upregulated under LDs and represses *HvFT1* and flowering in barley (Trevaskis et al., 2006; Hemming et al., 2008). *VRN-H2* shows a diurnal pattern of expression and is not expressed under SD conditions. The repression of *VRN-H2* under SDs is controlled by components of the circadian clock. Mutations in the barley clock gene *EARLY FLOWERING 3* (*HvELF3*) resulted in the expression of *VRN-H2* under SD conditions (Turner et al. 2013). Barley *hvelf3* mutants exhibited an early flowering phenotype independently of the photoperiod due to elevated expression levels of *Ppd-H1* and consequently *HvFT1* (Faure et al., 2012). SD expression of *VRN2* was also reported in the day-neutral *Ppd-D1a* wheat mutant which carries a deletion in the promoter of *Ppd-D1a* associated with constitutive expression of the gene (Turner et al., 2013). Similarly, in rice *Ghd7* and *OsPRR37*, homologous to *VRN2* and *PPD1*, exhibited epistatic interactions in the control of flowering time of rice populations grown in the field under different photoperiods (Shibaya et al., 2011; Fujino and Sekiguchi 2005). These studies in rice and wheat suggested that *PPD1/OsPRR37* and *VRN2/Ghd7* might interact, however, the mechanism that controls the activation of *VRN2* expression in response to photoperiod remains unclear.

Allelic variation of the two LD response genes *Ppd-H1* and *VRN-H2* has significantly contributed to the spread of barley cultivation across different environments. A natural mutation in the CCT domain of *Ppd-H1* is associated with lower transcript levels of *HvFT1* and delayed flowering under LDs compared to the wild type *Ppd-H1* allele, but is not associated with flowering variation under SDs (Turner et al., 2005; Hemming et al., 2008, Laurie et al., 1995; Decousset et al., 2000). The natural

mutation at *Ppd-H1* is prevalent in spring barley which is characterized by deletions of the *VRN-H2* locus and does not require vernalization (Dubcovsky et al., 2005). In winter barley, *VRN-H2* is downregulated during vernalization by *VRN-H1*, an *APETALA1/FRUITFUL* (*AP1/FUL*)-like MADS box transcription factor that is induced by vernalization (Trevaskis et al., 2006; Hemming et al., 2008, Alonso-Peral et al., 2011). Variation in the regulatory region of *VRN-H1* determines the timing and cold-dependency of *VRN-H1* activation and thus repression of *VRN-H2* (Hemming et al., 2008; 2009). In the LD cereals wheat and barley, the vernalization and photoperiod response pathways are known to converge on *FT1* (Trevaskis et al., 2007, Hemming et al., 2008). However, a recent study has identified potential epistatic interactions between *VRN-H2* and *HvCO1* in a nested association mapping population (Maurer et al., 2015). Putative interactions of *VRN-H2* with *Ppd-H1* and with *HvCO1* suggest that *VRN-H2* might also be important for the integration of photoperiod and vernalization signals.

The objectives of this study were to characterize the potential role of *HvCO2* in the control of flowering time under different photoperiods and to test if *HvCO1/CO2* genetically interact with *Ppd-H1* and *VRN-H2* to control flowering in barley. We show that *HvCO2* overexpression accelerates flowering in spring barley but does not abolish plant sensitivity to inductive LDs. Overexpression of *HvCO1* and *HvCO2* upregulated the expression of *VRN-H2*, which was associated with a delay in flowering under LD and SD conditions as compared to spring transgenic genotypes with a deletion of *VRN-H2*. In addition, variation at *Ppd-H1* controlled *VRN-H2* expression. Our data thus suggest that the floral activators *HvCO1/CO2* and *Ppd-H1* indirectly repress flowering before vernalization by controlling expression of *VRN-H2* under LDs.- These findings unravel a degree of functional conservation between *HvCO1/CO2* and *Ppd-H1* and their rice orthologs *Hd1* and *OsPRR37*, which function as floral repressors under LDs.

Results

Overexpression of *HvCO2* accelerated flowering time in a spring barley background

The effect of *HvCO2* on time to flowering was investigated by ectopically overexpressing the gene in the spring variety Golden Promise and analyzing flowering time and expression of major flowering time genes under LDs and SDs.

Under LDs, transgenic *Ubi::HvCO2* lines flowered on average 36 days after emergence (DAE) and thus significantly earlier than the null segregants and Golden Promise (WT) which required on average 54 days to flower (Figure 1). Under SD conditions, overexpression of *HvCO2* induced flowering, whereas the null segregants and the WT had not flowered by 150 DAE, when the experiment was stopped. *Ubi::HvCO2* lines flowered on average 78 DAE under SDs and thus significantly later than under LDs.

To further characterize the day length dependent effects of *Ubi::HvCO2* on flowering time, we evaluated the expression of *HvCO2*, *HvCO1* and of major flowering time genes such as *Ppd-H1*, *HvFT1*, *HvFT3*, and *VRN-H1* in leaf tissue of *Ubi::HvCO2* lines and the wild type controls under LD and SD conditions. Expression of *HvCO2* was significantly upregulated in the transgenic lines compared to the null segregants and the WT under LD and SD conditions (Figure 2A). Expression of *HvCO1* was significantly reduced in all *Ubi::HvCO2* lines compared with the null segregants and the WT under LDs. Under SDs, expression of *HvCO1* was below the detection limit at the time point when the seedlings were sampled (Figure 2B).

Expression of *HvFT1* was significantly upregulated in all tested transgenic lines under LDs, but was below the detection level in the null segregants and WT (Figure 2C). Under SD conditions however, expression of *HvFT1* was not detected in any of the tested genotypes. Expression of *HvFT3* was not different between transgenic and non-transgenic plants under LDs but was downregulated in all four *Ubi::HvCO2* lines as compared to the WT and the null segregants under SDs (Figure 2D). In addition, overexpression of *HvCO2* caused a significant upregulation of *VRN-H1* under LDs, whereas differences in *VRN-H1* expression between *Ubi::HvCO2* lines and the WT and the null segregants were not consistent under SDs (Figure. 2E). Expression levels of *Ppd-H1* in the *Ubi::HvCO2* lines did not significantly differ from those in non-transgenic controls under LDs and SDs (Figure 2F).

Taken together, overexpression of *HvCO2* caused early flowering under LD and SD conditions. However, transgenic lines showed a strong response to the photoperiod and this was associated with the photoperiod-dependent regulation of the barley flowering time genes *HvFT1* and *VRN-H1*.

Overexpression of *HvCO2* did not overcome the vernalization requirement

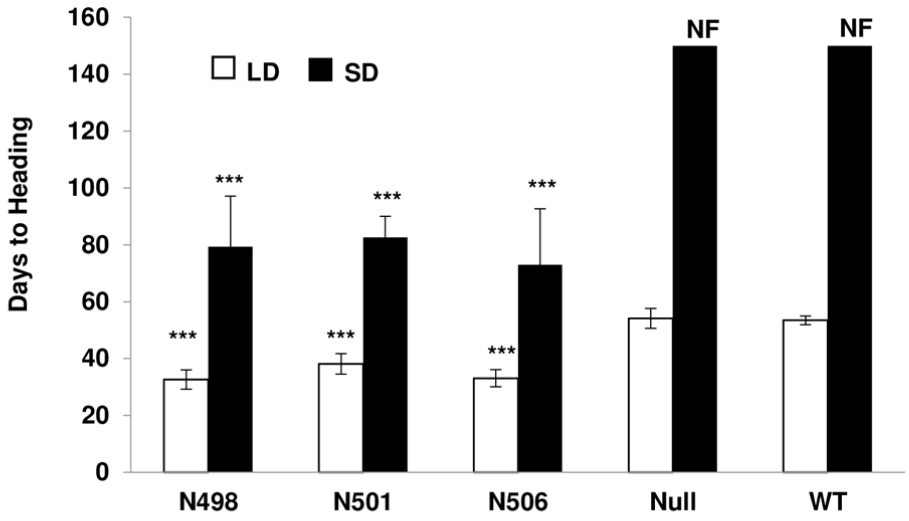

Figure 1. Analysis of flowering time of *Ubi::HvCO2* transgenic lines under long-day (LD) and short-day (SD) conditions.

Flowering time of *Ubi::HvCO2* transgenic lines (N498, N501 and N506), the null segregant line (Null) and Golden Promise (WT) grown under LD (white bars, 16h light) and SD (black bars, 8h light) conditions. Flowering time was measured for 5-20 plants for each of the *Ubi::HvCO2* lines, Null and WT in days from germination until heading. Null and WT did not flower at the end of the experiment (NF; 150 days) under SDs. Columns represent the average flowering time. Error bars: Standard deviation. *** refers to a significant difference in flowering time of the transgenic lines compared to the Null and the WT at $p<0.001$.

The genetic interactions of *HvCO2* with the photoperiod gene *Ppd-H1* and the vernalization genes *VRN-H1* and *VRN-H2* were evaluated by recording flowering time in an F_2 population derived from a cross between *Ubi::HvCO2* line (N506) and the winter variety Igri. The *Ubi::HvCO2* line (N506) in the background of the spring barley Golden Promise carries a natural mutation at *Ppd-H1*, a deletion of the *VRN-H2* locus, a deletion in the first regulatory intron of *VRN-H1* and a functional *HvFT3* gene. As a consequence, this genotype does not require vernalization and shows a reduced photoperiod response. In contrast, Igri is characterized by the wild type allele at *Ppd-H1*, winter alleles at V*RN-H1* and *VRN-H2* and a partial deletion of *HvFT3*. Consequently, Igri requires vernalization to flower and shows a strong photoperiod response. To test whether overexpression of *HvCO2* can overcome the vernalization requirement, F_2 plants were grown without vernalization under LDs and scored for flowering time.

Flowering time varied between 23 and 130 days in the F_2 population of *Ubi::HvCO2* x Igri under LDs (Figure S1). The F_2 population showed transgressive segregation as 37 plants (19%) flowered earlier than the average flowering time (41 d) of the transgenic parent. Only five plants (3%) flowered later than the winter parent Igri which flowered after 116 days.

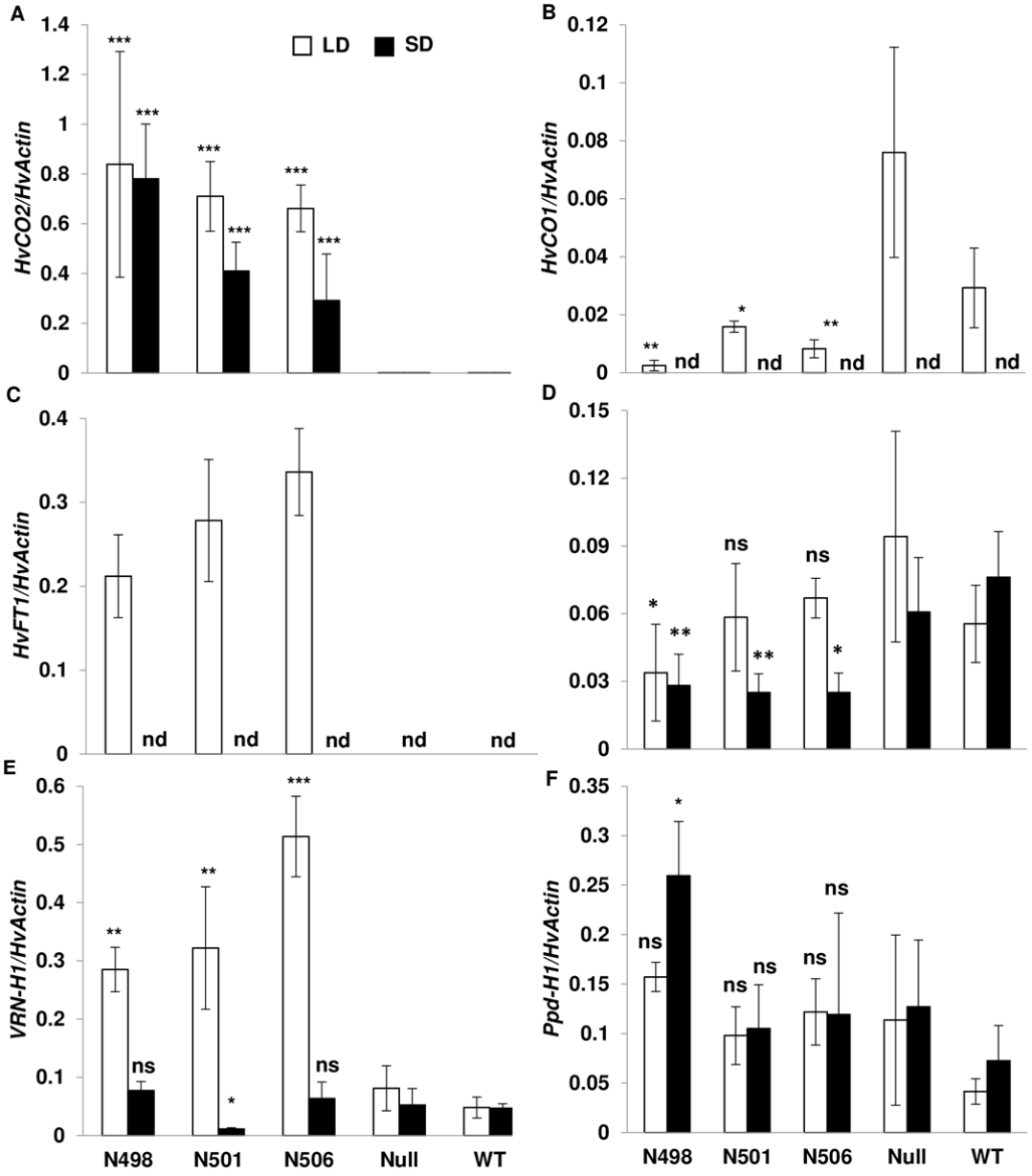

Figure 2. Expression levels of flowering time genes in *Ubi::HvCO2* transgenic lines.
Expression levels of flowering time genes in *Ubi::HvCO2* transgenic lines (N498, N501 and N506), null segregants (Null) and Golden Promise (WT) under LD (white bars, 16h light) and SD (black bars, 8h light) conditions. Expression analysis was performed on leaf samples collected two hours before the end of the light period at day 7 after germination under LDs and SDs. For each transgenic line, Null and WT 3-7 plants were used as biological replicates. Columns represent the average expression of **A)** *HvCO2*, **B)** *HvCO1*, **C)** *HvFT1*, **D)** *HvFT3*, **E)** *VRN-H1* **F)** *Ppd-H1* all normalized to the expression level of *HvActin*. nd: no expression detected. Error bars: Standard deviation. *, **, *** refers to a significant expression difference in the transgenic lines compared to the Null and the WT at p<0.05, p<0.01 and p<0.001, respectively. ns: no significant difference in expression at P<0.05. Statistical comparisons were performed separately for gene expression under LDs and SDs.

We associated genetic variation at the flowering time genes, *Ubi::HvCO2*, *Ppd-H1*, *VRN-H1*, *VRN-H2* and *HvFT3* with time to flowering in the F_2 population, to estimate the contribution of each of the tested genes to the overall trait variation. To analyze the genetic interaction of *Ubi::HvCO2* and *Ppd-H1* in the absence of *VRN-H2*, we also associated the allelic variation of the candidate genes with

206 flowering time in the spring/facultative F_2 subpopulation comprising all F_2 genotypes with a deletion
207 of the *VRN-H2* locus. We designated alleles segregating in the F_2 population and derived from the
208 winter parent with W (winter) and alleles derived from the spring barley Golden Promise with S
209 (spring).

210 In total, the overexpression of *HvCO2* and allelic variation at *VRN-H1*, *VRN-H2*, and *Ppd-H1*
211 accounted for 89% of the variation identified for flowering time in the F_2 population grown under LDs
212 (Table S1). Natural variation at *HvFT3* did not show any significant effect on flowering time under
213 LDs. The transgene *Ubi::HvCO2* accelerated flowering time, but explained only 16% of the overall
214 phenotypic variation (Figure 3A, Table S1). In contrast, natural variation at *VRN-H2* had the strongest
215 effect on flowering time and accounted for 51% of the flowering time variation (Figure 3B, Table S1).
216 F_2 genotypes carrying the winter allele of *VRN-H2* flowered on average after 74 days, and thus 42
217 days later than those carrying the deletion (spring allele) of the gene. The vernalization gene *VRN-H1*
218 explained 11% of the variation in days to flowering, as the winter allele delayed flowering time by on
219 average 18 days. Furthermore, the interaction between *VRN-H1* and *VRN-H2* was significant and
220 explained 3% of the phenotypic variation. The combination of winter alleles at *VRN-H2* and *VRN-H1*
221 delayed flowering time by additional 22 days compared with the sum of the effects of the winter
222 alleles at both genes. The vernalization genes *VRN-H1* and *VRN-H2* and their interaction thus
223 explained in total 65% of flowering time variation in the population. Consequently, the effects of
224 *VRN-H2* and *VRN-H1* had more pronounced effects on time to flowering than *Ubi::HvCO2*.
225 Nevertheless, *Ubi::HvCO2* reduced days to heading in the winter F_2 plants with homozygous and
226 heterozygous winter alleles at *VRN-H1* and *VRN-H2*, respectively, by about 22 days (Figure S2A).
227 Allelic variation at the major photoperiod gene *Ppd-H1* explained 5% of the overall variation in days
228 to flowering. The photoperiod-responsive allele reduced time to flowering by eight days compared to
229 the mutated *ppd-H1* allele. In spring or facultative F_2 genotypes with a deletion of *VRN-H2*, *Ppd-H1*
230 exerted the strongest effect on flowering time (65%, Table S2) even in the presence of the transgene
231 (Figure S2B). The wild type *Ppd-H1* allele accelerated flowering time by 11 days as compared to the
232 mutated *ppd-H1* allele in the transgenic F_2 genotypes with a deletion of *VRN-H2*.

233 Taken together, the repressive effect of *VRN-H2* was stronger than the effect of *Ubi::HvCO2* on
234 flowering. Nevertheless, the presence of the transgene accelerated flowering time also in the winter
235 genotypes. In transgenic F_2 genotypes with a deletion of the *VRN-H2* locus, variation at *Ppd-H1* had
236 the strongest effect on flowering time, consistent with the observation that transgenic genotypes
237 maintained a strong photoperiod response.

238 **Overexpression of *HvCO2* upregulated the floral repressor *VRN-H2***

239 To further characterize the molecular control of flowering time in the F_2 population, we analyzed the
240 effects of *Ubi::HvCO2* on expression levels of selected flowering time regulators.

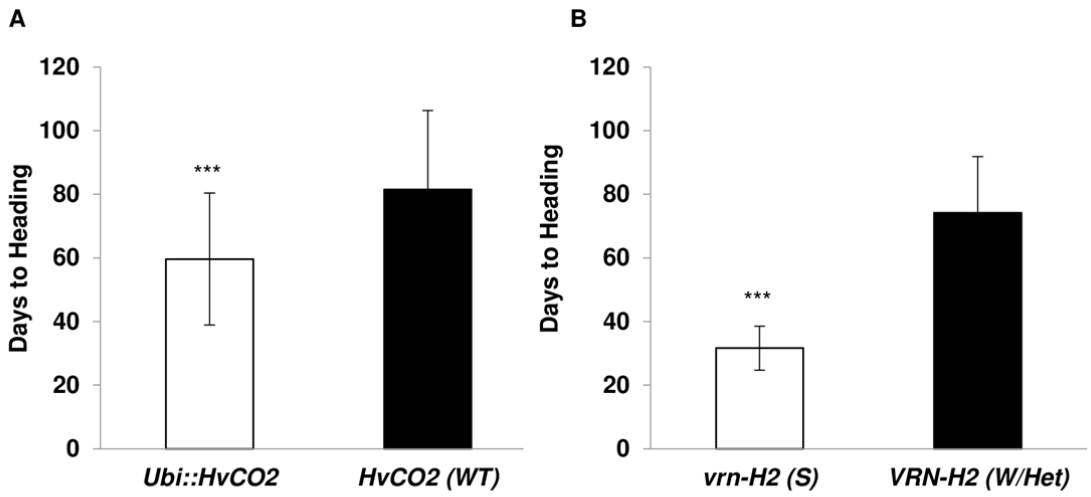

Figure 3. Effects of *Ubi::HvCO2* and *VRN-H2* on flowering time of the F_2 population *Ubi::HvCO2* x Igri under LD.

Columns represent the average flowering time of F_2 genotypes classified according to **(A)** the presence/absence of the transgene *Ubi::HvCO2* and **(B)** the allelic variation of *VRN-H2*. S: spring allele, W/Het: homozygous and heterozygous winter allele. Error bars: standard deviation. *** refers to a significant difference at P<0.001.

HvCO2 expression in F_2 genotypes carrying the transgene was on average 1000 times higher than in the non-transgenic F_2 genotypes (Figure 4A). Accordingly, the presence/absence of the transgene explained 72% of the variation in *HvCO2* expression (Table S3). Interestingly, the presence of *VRN-H2* was associated with a significant downregulation of *HvCO2* expression in F_2 genotypes carrying the WT *HvCO2* gene (Figure S3). *HvCO2* expression levels showed a high negative correlation with days to flowering (-0.58, Table S4). In addition, *HvCO2* exhibited a high positive correlation with expression levels of *Ppd-H1* (0.60) in the winter F_2 population, but not in spring F_2 population (Table S5).

Across the entire population, genetic variation at *VRN-H2*, *HvCO2* and their interactions explained 61, 6 and 15% of the variation in *VRN-H2* expression, respectively (Table S3). Interestingly, winter F_2 genotypes carrying the *Ubi::HvCO2* transgene showed on average an eleven times higher expression of *VRN-H2* than F_2 genotypes without the transgene (Figure 4B). Accordingly, expression levels of *HvCO2* and *VRN-H2* were highly correlated (0.79) in winter F_2 genotypes (Table S5). Despite the strong upregulation of the flowering repressor *VRN-H2* in the presence of *Ubi::HvCO2*, transgenic winter F_2 genotypes flowered earlier than the non-transgenic winter F_2 genotypes (Figure S2A). In addition, *VRN-H2* was significantly upregulated by the wild type allele of *Ppd-H1* in the background of non-transgenic F_2 genotypes (Figure 5A). The presence of *VRN-H2* in turn correlated with the downregulation of *Ppd-H1*, in particular in the background of the non-transgenic genotypes (Figure 5B).

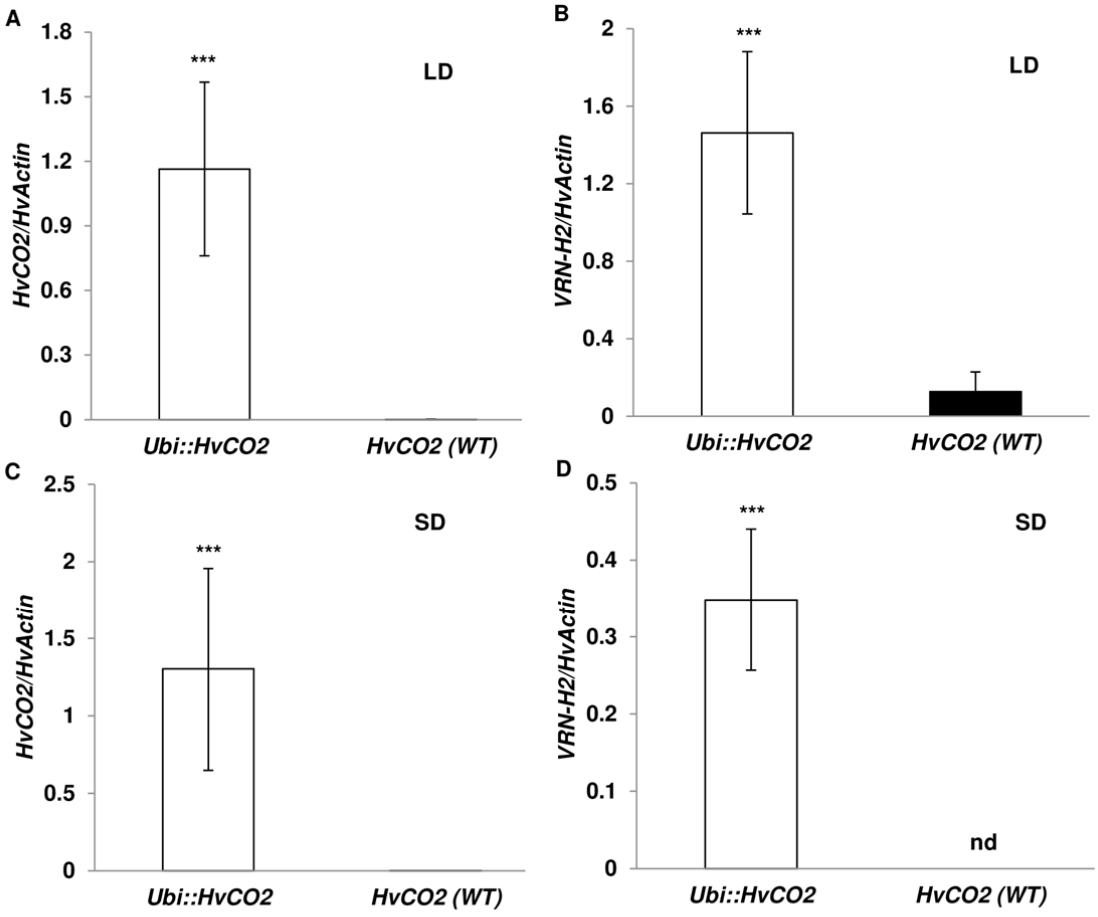

Figure 4. Effect of *Ubi::HvCO2* on expression of *HvCO2* and *VRN-H2* in F_2 genotypes of the population *Ubi::HvCO2* x Igri grown under LD and SD.

Columns represent the average expression of *HvCO2* (**A, C**) and *VRN-H2* (**B, D**), each normalized to *HvActin* in F_2 genotypes classified according to the presence/absence of the transgene *Ubi::HvCO2,* under long day (LD, 16h light, **A, B**) and short day (SD, 8h light, **C, D**). F_2 genotypes homozygous for the spring *Vrn-H2* allele were not considered in **B** and **D.** Expression analysis was performed on leaf samples collected two hours before the end of the respective light period at day 7 after germination. nd: no expression detected. Error bars: standard deviation. *** refers to a significant difference at $p<0.001$.

Allelic variation at *VRN-H2* exerted a strong effect on *HvFT1* expression levels. In the presence of *VRN-H2*, *HvFT1* expression was completely repressed in all F_2 genotypes independent of the transgene (Figure S4). On the other hand, F_2 genotypes with the *Ubi::HvCO2* transgene showed higher expression levels of *HvFT1* in the F_2 genotypes with a deletion of the *VRN-H2* locus (Figure 6B). Accordingly, across the entire population, variation in *HvFT1* expression was mainly controlled by *VRN-H2* (35%) and *Ubi::HvCO2* (22%, Table S3). Interestingly, the photoperiod responsive allele of *Ppd-H1* significantly upregulated the expression of *HvFT1* in the spring/facultative F_2 genotypes with a deletion of *VRN-H2*, in the presence of the transgene *Ubi::HvCO2* (Figure S5). *HvFT1* expression levels strongly correlated with days to flowering (-0.70) and with expression levels of *VRN-H1* (0.57), *Ppd-H1* (0.33) and *VRN-H2* (-0.47, Table S4).

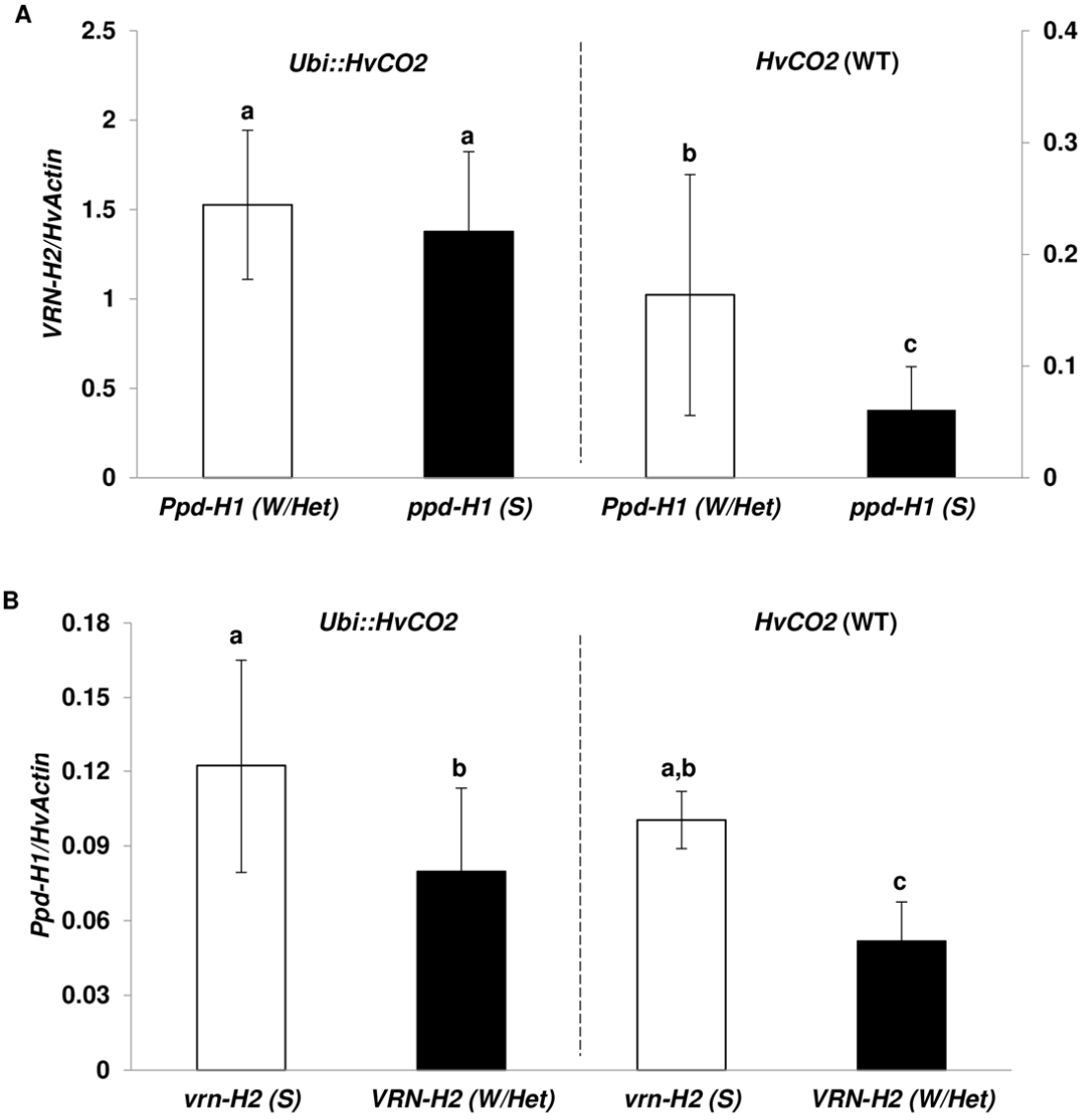

Figure 5. Reciprocal interaction between *Ppd-H1* and *VRN-H2* in the F_2 population *Ubi::HvCO2* x Igri under long day.

Columns represent the average expression of *VRN-H2* normalized to *HvActin* in F_2 genotypes classified according to the presence/absence of *Ubi*::*HvCO2* and allelic variation at *Ppd-H1*. F_2 genotypes homozygous for the spring *VRN-H2* allele were not considered. **(B)** Columns represent the average expression of *Ppd-H1* normalized to *HvActin* in F_2 genotypes classified according to the presence/absence of *Ubi::HvCO2* and *VRN-H2*. S: spring allele, W/Het: homozygous and heterozygous winter allele. Expression analysis was performed on leaf samples collected two hours before the end of the light period in long day (LD, 16h light) at day 7 after germination. Error bars: standard deviation. Letters on top of each graph indicate significant differences in expression levels at $p<0.05$.

In summary, *Ubi::HvCO2* caused a strong upregulation of *VRN-H2*. In addition, variation at *Ppd-H1* affected *VRN-H2* expression in the non-transgenic F_2 subpopulation. *VRN-H2* in turn was involved in the downregulation of *Ppd-H1* and *HvCO2*. Our findings thus suggest strong reciprocal interactions between *HvCO2*, *Ppd-H1* and *VRN-H2*. *Ubi::HvCO2* and *Ppd-H1* exhibited additive effects on *HvFT1* expression in the absence of *VRN-H2*.

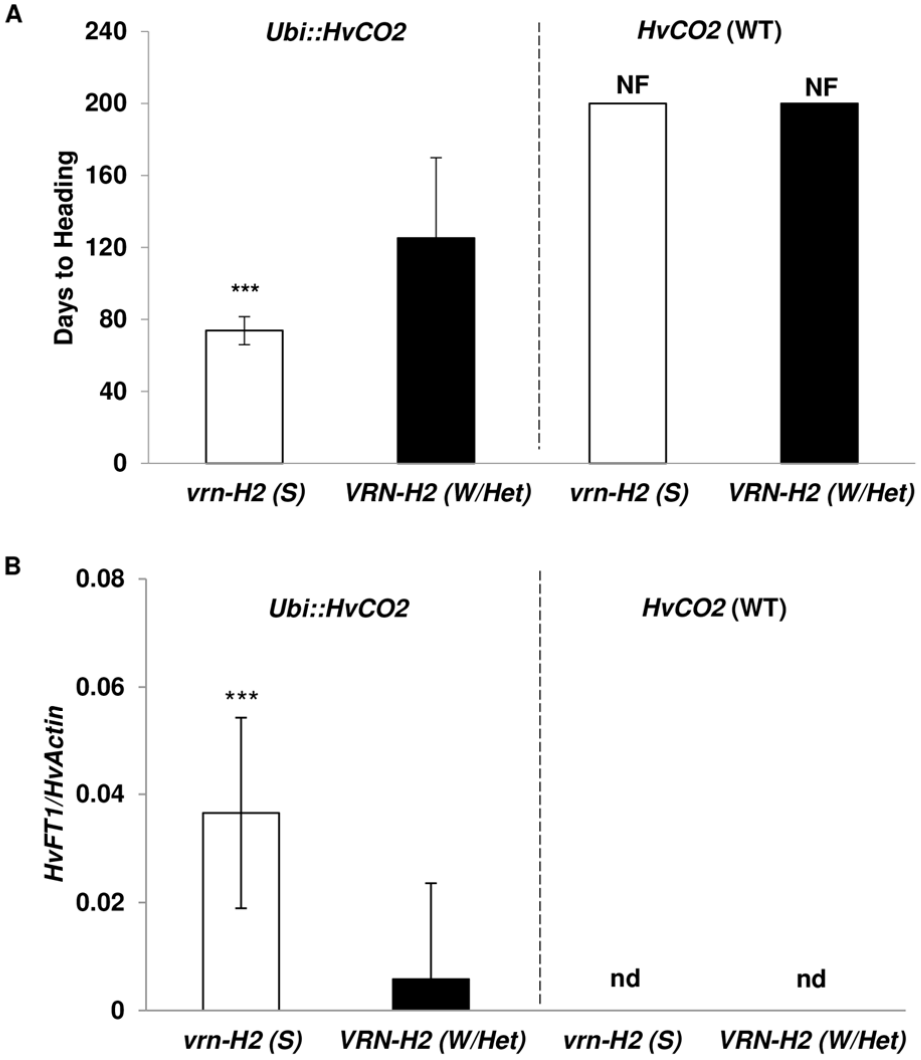

Figure 6. Effects of *Ubi::HvCO2* and allelic variation of *VRN-H2* on flowering time and *HvFT1* expression in *Ubi::HvCO2* x Igri F_2 genotypes under SD conditions.

The F_2 genotypes are classified according to the presence/absence of *Ubi::HvCO2* and *VRN-H2*. S: spring allele, W/Het: homozygous and heterozygous winter allele. **(A)** Columns represent the average flowering time of the different genotypic classes. Non-transgenic F_2 genotypes did not flower at the end of the experiment (NF; 200 days). **(B)** Columns represent the average *HvFT1* expression in F_2 genotypes classified according to variation at *Vrn-H2* and presence/absence of the transgene. Expression was analyzed in leaf samples harvested two hours before the end of the light period. nd: no expression detected. Error bars: standard deviation. *** refers to a significant difference at P<0.001.

Overexpression of *HvCO2* and *HvCO1* induced expression of *VRN-H2* and *HvFT1* under SD conditions

As the overexpression of *HvCO2* upregulated the expression of *VRN-H2* under LDs, we further tested if *Ubi::HvCO2* also upregulated *VRN-H2* expression under SDs, when the gene is usually not expressed. For this purpose, 168 F_2 genotypes derived from the cross *Ubi::HvCO2* x Igri were grown in the greenhouse under SD (8h-10h light) conditions and scored for flowering time and gene expression.

Overexpression of *HvCO2* caused an upregulation of *VRN-H2* in the transgenic F_2 genotypes also under SDs at 7 DAE, while no *VRN-H2* expression was detected in the non-transgenic F_2 genotypes (Figure 4C, D). Transgenic F_2 genotypes with the *VRN-H2* locus flowered on average after 125 DAE, while transgenic F_2 genotypes with a deletion of *VRN-H2* required on average 74 days to flower under SDs (Figure 6A). All non-transgenic F_2 genotypes failed to flower up to 200 DAE (when the experiment was stopped). Expression of *VRN-H2* as mediated by *Ubi::HvCO2* was thus associated with a significant delay in flowering also under SDs. Accordingly, *Ubi::HvCO2* and *VRN-H2* explained 48% and 11% of the observed variation in flowering time (Table S6). Expression levels of *HvFT1* were under the detection limit at 7 DAE, but were later (75 DAE) detected in transgenic F_2 genotypes under SDs (Figure 6B). Transgenic F_2 genotypes with a deletion of *VRN-H2* had six-fold increased expression levels of *HvFT1* as compared to their siblings with the winter allele of *VRN-H2*. Expression of *HvFT1* was not detected in the non-transgenic F_2 genotypes. Variation in *HvFT1* expression was thus mainly controlled by *VRN-H2* (29%) and *Ubi::HvCO2* (16%, Table S6). Finally, variation at *Ppd-H1* affected flowering time and *HvFT1* expression in the transgenic F_2 genotypes under SDs, when *Ppd-H1* does usually not have any effect on time to flowering (Table S6).

We further tested if overexpression of *HvCO1*, as the closest homolog of *HvCO2*, could also influence *VRN-H2* expression under LD and non-inductive SD conditions. The upregulation of *HvCO1* expression in *Ubi::HvCO1* x Igri F_2 genotypes carrying the *Ubi::HvCO1* transgene was associated with an upregulation of *VRN-H2* under LDs and SDs (Figure S6).

Taken together, *Ubi::HvCO1* and *Ubi::HvCO2* upregulated the expression of *VRN-H2* under LDs and SDs. Upregulation of *VRN-H2* in *Ubi::HvCO2* genotypes under SDs was associated with a delay in flowering time and a reduction in *HvFT1* expression as compared to *Ubi::HvCO2* genotypes with a deletion of *VRN-H2*. Finally, variation at *Ppd-H1* affected time to flowering and *HvFT1* expression in transgenic, but not WT F_2 genotypes under SDs.

Discussion

Overexpression of *HvCO2* causes photoperiod-dependent early flowering in barley

In *A. thaliana, CO* is an important promoter of flowering in response to LD (Koornneef et al., 1991; Putterill et al., 1995). Arabidopsis plants constitutively overexpressing *CO* were early flowering and almost completely insensitive to day-length (Onouchi et al., 2000). In the current study, overexpression of *HvCO2*, which represent with *HvCO1* the closest barley orthologs of *AtCO* (Griffiths et al., 2003), also caused early flowering in spring barley under LDs and SDs. However, transgenic plants overexpressing *HvCO2* retained a strong response to day-length and flowered significantly earlier under LDs than under SDs. Accordingly, *HvFT1* upregulation in the transgenic lines occurred significantly later under SDs when compared to LD conditions. Analysis of flowering time and gene expression in the cross between *Ubi::HvCO2* x Igri suggested that the photoperiod response of transgenic lines was influenced by *Ppd-H1*. Variation at *Ppd-H1* affected flowering time and expression of *HvFT1* in transgenic spring F_2 genotypes under LDs. Consequently, *Ppd-H1* controlled flowering time downstream of *HvCO2* expression under LDs (Figure 7). Similarly, transgenic lines overexpressing *HvCO1* retained a photoperiod response, and *Ppd-H1* exerted a significant effect on flowering time in *Ubi::HvCO1* transgenic lines grown under LDs (Campoli et al., 2012). Consequently, both *HvCO1* and *HvCO2* accelerate flowering, but their effects are modified by day-length and natural variation at *Ppd-H1*. In contrast, upregulation of *Ppd-H1* expression in a barley mutant with a non-functional *HvELF3* gene was associated with photoperiod-independent early flowering (Faure et al., 2012). Moreover, natural mutations in the promoters of the homologous *PPD1* genes in wheat and consequent upregulation of *PPD1* expression caused photoperiod insensitivity and early flowering (Beales et al., 2007). These reports, together with our results indicate that expression variation of *Ppd1* has a stronger impact on photoperiodic flowering than expression changes of *CO1/CO2*. Similarly, a rice line with a non-functional allele at *Hd1* (rice *CO*) retained sensitivity to day-length and complete day-length insensitivity was only observed when alleles at both *Hd1* (rice *CO*) and *Hd2* (*OsPRR37*) were non-functional (Lin et al., 2000). On the other hand, variation at *PRR37* orthologs affected flowering time only in the background of a functional CO ortholog in Sorghum and rice (Lin et al., 2000; Yang et al., 2014). This suggests that the ability of *PRR37*-like genes to control flowering is dependent on CO. In barley, variation at *Ppd-H1* only affected flowering time under LDs, however, in the background of *Ubi::HvCO2* plants variation at *Ppd-H1* regulated *HvFT1* expression and influenced flowering time also under SDs. The effect of *Ppd-H1* on flowering time was thus modified by overexpression of *HvCO2* which suggested that also in barley, *Ppd-H1* interacted with *HvCO2*. In Arabidopsis, factors controlling CO protein stability have a strong impact on flowering time. The photoreceptors *CRY1/2* and *PHYA* stabilize CO whereas *PHYB* destabilizes the protein and accordingly, *cry2* and *phyA* mutants are late flowering while *phyB* mutants are early flowering (Turck et al., 2008). Interestingly, a *Ppd-H1* homolog in Arabidopsis; *PRR3,* was shown to

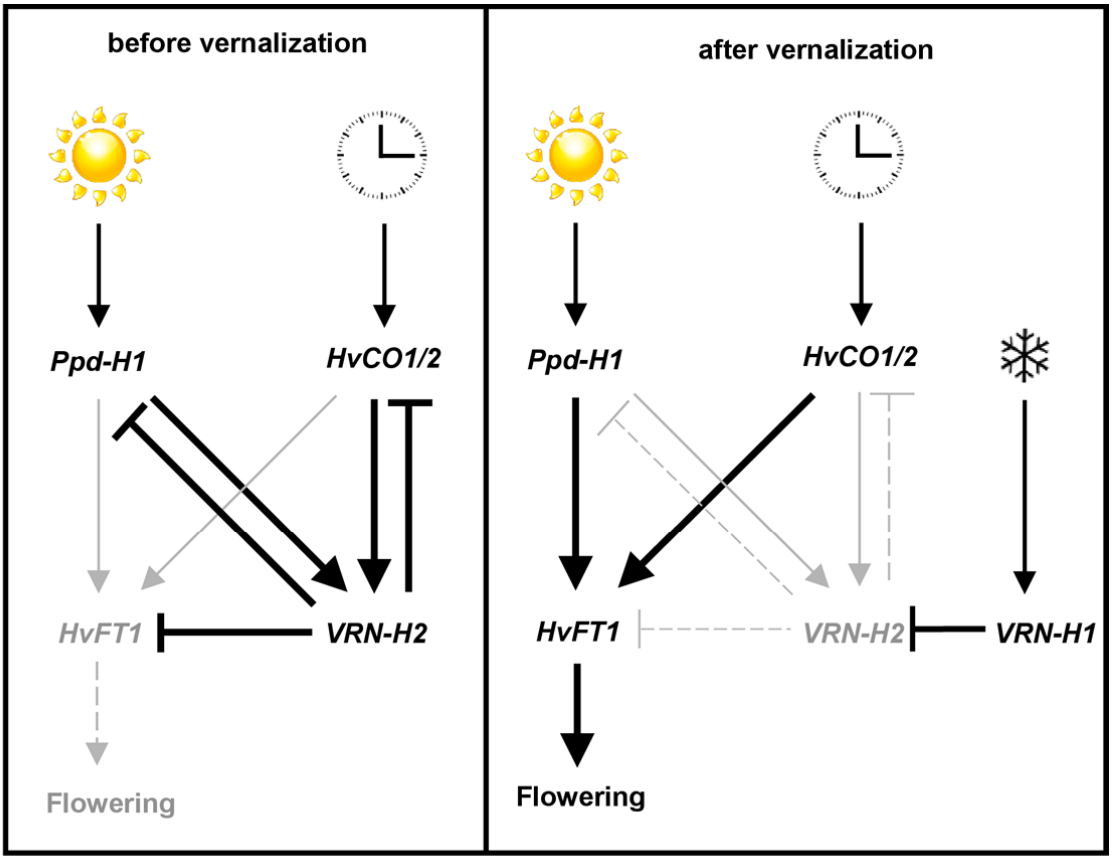

Figure 7. A model for the coregulation of *VRN-H2* and *HvFT1* by *HvCO1/CO2* and *Ppd-H1* in winter barley under LD conditions before and after vernalization. *HvCO1/CO2* and *Ppd-H1* induce the expression of *VRN-H2* which acts as a strong repressor of *HvFT1* and flowering time in winter barley before vernalization. *VRN-H2,* in turn, represses *HvCO1/CO2* and *Ppd-H1*. Upregulation of *VRN-H1* during vernalization represses *VRN-H2*. In the absence of *VRN-H2* after vernalization (or in spring barley), *Ppd-H1* and *HvCO1/CO2* upregulate *HvFT1* and induce flowering under LD conditions.

stabilize TOC1 protein which shares the CCT domain with CO (Para et al., 2007). The induction of *HvFT1* by *Ppd-H1* may thus be dependent on the posttranscriptional modification of *HvCO1/CO2*. The availability of barley lines with non-functional alleles at *HvCO1* and *HvCO2* would further help to dissect the genetic interactions of *Ppd-H1* and *HvCO1/CO2* in barley.

HvCO1/CO2 and *Ppd-H1* coregulate *VRN-H2* expression

In Arabidopsis, overexpression of the photoperiod response gene *CO* could largely overcome the delay in flowering caused by the overexpression of the major vernalization gene *FLC* (Hepworth et al., 2002). However, we could show that in barley flowering time was delayed by the winter alleles at *VRN-H2* and *VRN-H1* even in the presence of *Ubi::HvCO2*. Interestingly, overexpression of *HvCO1/CO2* caused an upregulation of the flowering repressor *VRN-H2* under inductive LDs, but also under SD conditions, when the gene is normally not expressed. *VRN-H2* was functional, repressed *HvFT1* expression and delayed flowering time under LDs and SDs. Consequently, *HvCO1/CO2* are involved in mediating the photoperiodic regulation of *VRN-H2*. As such, *HvCO1/CO2* acted as a promoter of flowering in a spring barley background, but as an indirect repressor of flowering in a

winter barley line with a functional *VRN-H2* gene. *Hd1,* the rice orthologue of *CO,* was also proposed to have these two opposite functions of repressing and promoting flowering by inhibiting and inducing *Hd3a* expression (*FT* orthologue) under LDs and SDs, respectively (Yano et al., 2000; Hayama et al., 2003). Consequently, the involvement of *CO* in LD repression of flowering seems to be partially conserved between rice and barley despite the opposite flowering behavior of the two cereal crops under LD conditions. In rice, floral repression under LDs is mediated by *Ghd7,* a CCT domain gene which like *Vrn-H2* is upregulated under LDs and represses expression of *Hd3a* (Xue et al., 2008). It was suggested that *Ghd7* and *Hd1* independently control the photoperiod response in rice (Tsuji et al., 2011, Xue et al., 2008). However, Saito et al. (2012) could show that *OsElf3* controlled the expression of both, *Hd1* and *Ghd7* and suggested that both genes may interact to control *Hd3a*. In addition, Shibaya et al. (2011) demonstrated that *Ghd7* interacted with *Hd2* which was identified as *OsPRR37*, the rice homolog of *Ppd-H1*. Interestingly, our expression analysis revealed that the functional allele of *Ppd-H1* was associated with higher expression levels of *VRN-H2* under LDs in the non-transgenic F_2 genotypes with a winter allele at *VRN-H2*. Although allelic variation at *Ppd-H1* has not yet been associated with *VRN-H2* expression levels, barley *hvelf3* and wheat *Ppd-D1a* mutants in which *Ppd-H1 and Ppd-D1* are constitutively expressed, upregulated *VRN-H2* under non-inductive SDs (Turner et al., 2013). Moreover, expression studies in wheat *PhyC* mutants revealed a correlated downregulation of *PPD1* and *VRN-H2*, (Chen et al., 2014). These findings indicate that *Ppd-H1* is involved in the regulation of *VRN-H2*. *Ppd-H1* may thus also act as an indirect repressor of flowering by upregulating *VRN-H2* under LDs before vernalization. We propose that before vernalization *Ppd-H1* functions as a floral repressor under LDs as has been shown for the rice and Sorghum orthologs of *Ppd-H1*, *OsPRR37* and *SbPRR37* (Koo et al., 2013; Murphy et al., 2011). Our results showed that functional allelic diversity at *Ppd-H1* and overexpression of *HvCO1/CO2* could influence *VRN-H2* expression (Figure 7). This supports our previous suggestion that *Ppd-H1* and *HvCO1/CO2* might interact posttranscriptionally to control downstream targets. However, we also observed that overexpression of *HvCO2* was associated with an upregulation of *Ppd-H1* under LDs, indicating that both genes may have also interacted transcriptionally. Furthermore, expression levels of *Ppd-H1* and *HvCO2* were repressed by *VRN-H2* indicating the presence of negative feedback loops from *VRN-H2* to *Ppd-H1* and *HvCO2*. Consequently, expression levels of the three genes were strongly interdependent. Each of the three genes encodes a protein with a CCT domain that is known to be important for the function of the protein and for protein-protein interactions (Robson et al., 2001; Turner et al., 2005; Yan et al., 2004; Distelfeld et al., 2009; Li et al., 2011). Li et al. (2011) demonstrated that the CCT domains of VRN2 and CO2 proteins in wheat interacted with the same set of HAP/NF-Y proteins. The authors suggested that the competitive interactions of VRN2 and CO2 with NF-Y proteins played an important role in the integration of seasonal signals for the transcriptional regulation of *VRN3* (*TaFT*) in wheat. HAP/NF-Y proteins are known to regulate flowering in Arabidopsis (Wenkel et al., 2006; Kumimoto et al., 2008; 2010) and rice (Wei et al., 2010; Yan et al., 2011; Dai et al., 2012). In addition, NF-Y

proteins are involved in plant responses to various environmental stresses, such as drought and osmotic stresses (Nelson et al., 2007, Stephenson et al., 2007; Li et al., 2008). Reciprocal transcriptional activation and repression of *CO*, *PPD1* and *VRN2* may help to prioritize environmental signals, whereas competitive interactions of these genes with HAP/NF-Y factors could provide a complex system to integrate the seasonal cues with multiple stress signals to fine-tune the regulation of flowering time.

Conclusion:

HvCO2 overexpression enhanced *HvFT1* expression and accelerated flowering time, but expression levels of *HvFT1* and day-length sensitivity were controlled by *Ppd-H1* downstream of *HvCO2* overexpression (Figure 7). *HvCO1/CO2* and *Ppd-H1* coregulated *HvFT1* but also *VRN-H2,* which revealed a dual function of *CO* orthologs and *Ppd-H1* as activator/repressor of flowering depending on the presence of *VRN-H2*. LDs repress flowering before vernalization through the function of *Ppd-H1*, *HvCO* and *VRN-H2*, but activates flowering after vernalization when *VRN-H2* is downregulated. Consequently, floral repression through *VRN-H2* and floral activation through *HvFT1* is regulated by the same set of genes, *Ppd-H1* and *HvCO*. Our work suggests that the LD repression of flowering by *PRR* and *CO* genes is conserved between rice and barley and possibly among other grasses. Finally, the genetic interactions between *HvCO* and *Ppd-H1* with *VRN-H2* are important to consider for cereal breeding programs as manipulation of the photoperiod response pathway does also affect the vernalization response.

Materials and Methods

Generation of transgenic *Ubi::HvCO2* lines and their growth conditions

Barley plants of the spring variety Golden Promise were transformed with an overexpression construct generated with the cDNA clones of *HvCO2* (AF490470) driven by the maize ubiquitin promoter (Christensen et al., 1992). The overexpression cassette was inserted into the pWBVEC8 binary vector (Wang et al., 1998) and introduced into *Agrobacterium tumefaciens*. *Agrobacterium*-mediated transformation was then performed on excised barley embryos (Tingay et al., 1997; Matthews et al., 2001).

Independent barley transformants were regenerated, and T1 and T2 plants were screened for the presence of the transgene using two pairs of primers that bind to the hygromycin selectable marker gene and the *HvCO2* cDNA sequence (Table S7). The generation of transgenic *Ubi::HvCO1* lines is described in Campoli et al. (2012).

Three independent transgenic T_2-families designated *Ubi::HvCO2* lines N498, N501 and N506, a null segregant control line that lost the transgene and the wild type Golden Promise (WT) were sown in

soil and grown under LD (16h light/8h dark) and SD (8h light/16h dark) in the greenhouse (temperature 20°C/16°C days/nights). Five to 20 plants of each of the transgenic line, the null segregants and the WT were used to score flowering time, which was measured in days from emergence until heading (days after emergence; DAE). Heading was scored as the spike awns emerged from the sheath of the main shoot flag leaf (Zadoks stage 49, Zadoks et al., 1974). Leaf material from three to seven plants (biological replicates) for each tested line was collected for RNA extraction and gene expression analysis. The samples were harvested 7 DAE two hours before the end of the light period under LDs and SDs (Zeitgeber time (ZT), ZT14 under LD and ZT6 under SD)

Generation of *Ubi::HvCO2* x Igri and *Ubi::HvCO1* x Igri F_2 populations and their growth conditions

For the generation of the F_2 populations, each of the transgenic lines *Ubi::HvCO2* N506 and *Ubi::HvCO1* N2330 were crossed with the winter barley Igri. Golden Promise (WT), the genetic background of the transgenic lines, carries the spring allele of *Ppd-H1* with a mutation in the CCT domain. This mutation causes reduced photoperiod sensitivity and delays flowering under LDs. In addition, the WT is characterized by a spring allele at *VRN-H1* and a deletion of the *VRN-H2* locus and consequently does not require vernalization for the induction of flowering. Finally, Golden Promise carries a functional *HvFT3* gene which accelerates development under SDs (Laurie et al., 1995; Faure et al., 2007). In contrast, Igri carries the dominant *Ppd-H1* allele with a strong photoperiod response and winter alleles at *VRN-H1* and *VRN-H2*, and thus needs vernalization to flower. Furthermore, Igri is characterized by a partial deletion of *HvFT3*. In the resulting F_2 populations, alleles derived from the winter parent Igri are designated with W and alleles derived from the spring parent Golden Promise with S.

One hundred and ninety-one F_2 plants and 168 F_2 plants derived from the cross *Ubi::HvCO2* x Igri were sown in soil and grown in the greenhouse (temperature20°C/16°C days/nights) under LD (16h/8h light/dark), and SD (8h light/16h dark) conditions, respectively. After 50 days in 8h-SD, the light period was extended to 10h to accelerate plant development. Seedlings were not subjected to vernalization and flowering time was scored as number of days from emergence until heading (Zadoks stage 49). Leaf material was harvested from parental lines and 71 F_2 genotypes 7 DAE at ZT14 under LDs and from all 168 F_2 genotypes 7 DAE at ZT6 under SDs and subsequently used for RNA extraction and gene expression analysis. The selection of F_2 genotypes for gene expression analysis under LDs was based on the genotypic information to balance the number of plants within each genotypic class at the analyzed flowering time genes (the transgene, *Ppd-H1, VRN-H1* and *VRN-H2*). Additional leaf samples for gene expression analysis were harvest from 55 F_2 genotypes grown under SDs 75 DAE (25d after extending the photoperiod to 10h). Selection of the genotypes was also based

on the genotypic information of the genotypes and excluded genotypes that had already flowered by the time of sampling.

Similarly, 80 F_2 genotypes derived from the cross *Ubi::HvCO1* x Igri were grown under the same LDs and SDs conditions. The F_2 genotypes were genotyped for the transgene *Ubi::HvCO1* and *VRN-H2*. Expression analysis was performed on a subset of 20 F_2 genotypes under LDs and 12 F_2 genotypes under SDs, which had been selected for the dominant winter allele *VRN-H2* but segregated for the presence of the transgene. Expression of *HvCO2* and *VRN-H2* was quantified in leaf samples harvested 22 and 11 DAE under LDs (ZT14) and SDs (ZT6), respectively.

DNA extraction and genotyping of the segregating populations

Genomic DNA of individual F_2 genotypes was extracted from leaf samples following the Biosprint DNA extraction protocol (Qiagen). F_2 genotypes of all analyzed populations were genotyped for the presence of the transgene and allelic diversity of the major flowering genes *Ppd-H1* (Turner et al., 2005), *VRN-H1* (Hemming et al., 2009), *VRN-H2* (Dubcovsky et al., 2005) and *HvFT3* (Faure et al., 2007, Kikuchi et al., 2009). Polymerase chain reactions (PCR) were performed as described in the original references (List of primers in Table S7).

RNA extraction, cDNA synthesis and quantitative real time PCR (qRT-PCR)

Total RNA extraction, first-strand cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCRs) for individual F_2 plants were performed as described in Campoli et al. (2012). qRT-PCRs were performed using gene-specific primers (Table S7). Two technical replicates were used for each cDNA sample and 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

The statistical significance of differences in flowering time and gene expression levels between each of the *Ubi::HvCO2* genotypes and the wild type and the null controls (WT + Null combined) grown under LDs and SDs was determined using Student's *t*-test. A fixed model analysis of variance (ANOVA) for unbalanced designs was used to calculate significant effects and two-way interaction effects of the transgene and allelic variation at *Ppd-H1, VRN-H1, VRN-H2* and *HvFT3* on flowering time and gene expression in all tested F_2 populations. Pearson correlation coefficients were calculated between flowering time and gene expression values in the tested populations.

Supplemental Material

Table S1. Analysis of variance (ANOVA) of flowering time of the F_2 population *Ubi::HvCO2* x Igri grown under LD (16h light) conditions.

Table S2. Analysis of variance (ANOVA) of flowering time of the spring/facultative subpopulation (genotypes without *VRN-H2*) of *Ubi::HvCO2* x Igri F_2 population grown under LD (16h light) conditions.

Table S3**.** Analysis of variance (ANOVA) for expression of flowering time genes in the F_2 population *Ubi::HvCO2* x Igri grown under LD (16h light) conditions. Expression analysis was performed on leaf samples taken two hours before the end of the light period at day 7 after emergence.

Table S4. Pearson correlation coefficients of flowering time (measured as days to heading) and expression levels of tested flowering genes in the F_2 population *Ubi::HvCO2* x Igri grown under LD (16h light) conditions. Expression analysis was performed on leaf samples taken two hours before the end of the light period at day 7 after emergence.

Table S5. Pearson correlation coefficients of flowering time (measured as days to heading) and expression levels of tested flowering genes in the spring/facultative (genotypes without *VRN-H2*, upper triangle) and winter (lower triangle) subpopulations of the F_2 population *Ubi::HvCO2* x Igri grown under LD (16h light) conditions. Expression analysis was performed on leaf samples taken two hours before the end of the light period at day 7 after emergence.

Table S6. Analysis of variance (ANOVA) of *HvFT1* expression and flowering time of the F_2 population *Ubi::HvCO2* x Igri grown under SD conditions. Expression analysis was performed on leaf samples taken two hours before the end of the light period at day 75 after germination (day 25 after transfer from 8h to 10h SD).

Table S7. List of primers used in this study.

Figure S1: Flowering time of the F_2 population *Ubi::HvCO2* x Igri under long-day (LD) conditions.

Figure S2: Effects of *Ubi::HvCO2* and *Ppd-H1* on flowering time in A) F_2 genotypes with a winter background (winter alleles at *VRN-H1* and *VRN-H2*) and B) transgenic F_2 genotypes with a spring or facultative background (with a deletion of the *VRN-H2* locus) grown under LD conditions.

Figure S3: Effects of *VRN-H2* on expression of *HvCO2* in the F_2 population *Ubi::HvCO2* x Igri under LD conditions.

Figure S4: Effects of *Ubi::HvCO2* and *VRN-H2* on expression of *HvFT1* in the F_2 population *Ubi::HvCO2* x Igri under LD conditions.

Figure S5: Effect of *Ppd-H1* on expression levels of *HvFT1* in transgenic spring/facultative F_2 genotypes *Ubi::HvCO2* x Igri under LD conditions.

Figure S6: Effects of *Ubi::HvCO1* on expression of *HvCO1* and *VRN-H2* in F_2 genotypes of the population *Ubi::HvCO1* x Igri grown under LD and SD conditions.

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Authors' Contribution

M.A.M. and M.K. conceived and designed the experiments. M.A.M. carried out all experiments and analyzed the data. M.A.M. and M.K wrote the manuscript.

Competing Interests

The authors do not have any financial, personal or professional interests that have influenced this present paper.

Figure Legends

Figure 1.

Analysis of flowering time of *Ubi::HvCO2* transgenic lines under long-day (LD) and short-day (SD) conditions.

Flowering time of *Ubi::HvCO2* transgenic lines (N498, N501 and N506), the null segregant line (Null) and Golden Promise (WT) grown under LD (white bars, 16h light) and SD (black bars, 8h light) conditions. Flowering time was measured for 5-20 plants for each of the *Ubi::HvCO2* lines, Null and WT in days from germination until heading. Null and WT did not flower at the end of the experiment (NF; 150 days) under SDs. Columns represent the average flowering time. Error bars: Standard deviation. *** refers to a significant difference in flowering time of the transgenic lines compared to the Null and the WT at $p<0.001$.

Figure 2.

Expression levels of flowering time genes in *Ubi::HvCO2* transgenic lines.

Expression levels of flowering time genes in *Ubi::HvCO2* transgenic lines (N498, N501 and N506), null segregants (Null) and Golden Promise (WT) under LD (white bars, 16h light) and SD (black bars, 8h light) conditions. Expression analysis was performed on leaf samples collected two hours before the end of the light period at day 7 after germination under LDs and SDs. For each transgenic line, Null and WT 3-7 plants were used as biological replicates. Columns represent the average expression of **A)** *HvCO2*, **B)** *HvCO1*, **C)** *HvFT1*, **D)** *HvFT3*, **E)** *VRN-H1* **F)** *Ppd-H1* all normalized to the expression level of *HvActin*. nd: no expression detected. Error bars: Standard deviation. *, **, *** refers to a significant expression difference in the transgenic lines compared to the Null and the WT at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. ns: no significant difference in expression at $P<0.05$. Statistical comparisons were performed separately for gene expression measured under LDs and SDs.

Figure 3.

Effects of *Ubi::HvCO2* and *VRN-H2* on flowering time of the F_2 population *Ubi::HvCO2* x Igri under LD conditions.

Columns represent the average flowering time of F_2 genotypes classified according to **(A)** the presence/absence of the transgene *Ubi::HvCO2* and **(B)** the allelic variation of *VRN-H2*. S: spring allele, W/Het: homozygous and heterozygous winter allele. Error bars: standard deviation. *** refers to a significant difference at $P<0.001$.

Figure 4.

Effect of *Ubi::HvCO2* on expression of *HvCO2* and *VRN-H2* in F_2 genotypes of the population *Ubi::HvCO2* x Igri grown under LD and SD conditions.

Columns represent the average expression of *HvCO2* **(A, C)** and *VRN-H2* **(B, D)**, each normalized to *HvActin* in F_2 genotypes classified according to the presence/absence of the transgene *Ubi::HvCO2,* under long day (LD, 16h light, **A, B**) and short day (SD, 8h light, **C, D**). F_2 genotypes with the deleted *VRN-H2* locus were not considered in **B** and **D**. Expression analysis was performed on leaf samples collected two hours before the end of the light period at day 7 after germination. nd: no expression detected. Error bars: standard deviation. *** refers to a significant difference at $p<0.001$.

Figure 5.

Reciprocal interaction between *Ppd-H1* and *VRN-H2* in the F_2 population *Ubi::HvCO2* x Igri under long day.

(A) Columns represent the average expression of *VRN-H2* normalized to *HvActin* in F_2 genotypes classified according to the presence/absence of *Ubi*::*HvCO2* and allelic variation at *Ppd-H1.* WT/Het: homozygous and heterozygous wild type allele, M: mutant allele. F_2 genotypes with the deleted *VRN-H2* locus were not considered. **(B)** Columns represent the average expression of *Ppd-H1* normalized to *HvActin* in F_2 genotypes classified according to the presence/absence of *Ubi::HvCO2* and *VRN-H2*. S:

spring allele, W/Het: homozygous and heterozygous winter allele. Expression analysis was performed on leaf samples collected two hours before the end of the light period in long day (LD, 16h light) at day 7 after germination. nd: no expression detected. Error bars: standard deviation. Letters on top of each graph indicate significant differences in expression levels at $p<0.05$.

Figure 6.

Effects of *Ubi::HvCO2* and allelic variation of *VRN-H2* on flowering time and *HvFT1* expression in *Ubi::HvCO2* x Igri F_2 genotypes under SD conditions.

The F_2 genotypes are classified according to the presence/absence of the overexpressed *HvCO2* and allelic variation of *VRN-H2*. S: spring allele, W/Het: homozygous and heterozygous winter allele. **(A)** Columns represent the average flowering time of the different genotypic classes. Non-transgenic F_2 genotypes did not flower at the end of the experiment (NF; 200 days). **(B)** Columns represent the average *HvFT1* expression of the different genotypic classes normalized to the expression of *HvActin*. Expression was analyzed in leaf samples harvested two hours before the end of the light period from a subset of F_2 genotypes which did not flower by the date of sampling (75 DAE). nd: no expression detected. Error bars: standard deviation. *** refers to a significant difference at $P<0.001$.

Figure 7.

A model for the coregulation of *VRN-H2* and *HvFT1* by *HvCO1/CO2* and *Ppd-H1* in winter barley under LD conditions before and after vernalization. *HvCO1/CO2* and *Ppd-H1* induce the expression of *VRN-H2* which acts as a strong repressor of *HvFT1* and flowering time in winter barley before vernalization. Upregulation of *VRN-H1* during vernalization represses *VRN-H2*. In the absence of *VRN-H2* after vernalization (or in spring barley), *Ppd-H1* and *HvCO1/CO2* upregulate *HvFT1* and induce flowering under LD conditions.

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