CONSTANS Controls Floral Repression by Up-Regulating *VERNALIZATION2* (*VRN-H2*) in Barley[1]

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In barley (*Hordeum vulgare*), *PHOTOPERIOD1* (*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 (*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-H2* locus, overexpression of *HvCO1/CO2* caused an up-regulation 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 down-regulation 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 the 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.

Flowering is one of the most critical stages in the life cycle of plants. The 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 daylength or photoperiod (Greenup et al., 2009). Long photoperiods promote flowering in the model and facultative long-day (LD) plant Arabidopsis (*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 (*CONSTANS, CONSTANS*-like, and *TIMING OF CAB EXPRESSION1* [*TOC1*]) domain (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 (LDs) but after dusk in short days (SDs; 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 PHOTOMORPHO-GENIC1 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 date1* (*Hd1*), the rice ortholog of *CO*, promotes flowering by inducing the expression of *Hd3a*, the ortholog of *FT* (Izawa et al., 2002; Kojima et al., 2002). Under LDs, however, *Hd1* represses flowering through the down-regulation 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 (*Triticum aestivum*), Brachypodium (*Brachypodium distachyon*) 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 an LD activator of flowering, as seen in Arabidopsis, or an 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 to trans-acting regulatory mechanisms.

In rice, LD repression of flowering is mediated by two additional CCT domain genes: *Hd2/PSEUDO-RESPONSE REGULATOR37* (*OsPRR37*) and *Hd4/GRAIN NUMBER, PLANT HEIGHT, AND HEADING DATE7* (*Ghd7*; Xue et al., 2008; Koo et al., 2013; Gao et al., 2014). *OsPRR37* is orthologous to the Arabidopsis circadian clock gene *PRR3/7* and is characterized by a pseudoreceiver 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, *PHOTOPERIOD1* (*Ppd-H1*), the barley homolog of the LD repressor *OsPRR37*, is the major photoperiod response gene in barley and induces flowering under LDs by up-regulating *HvFT1*, the barley homolog of *Hd3a* (Turner et al., 2005). Barley carries five *FT* homologs, of which *HvFT1* correlates with flowering time under LD conditions, while a natural deletion at *HvFT3* has been associated with floral development under SD 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; Campoli et al., 2012; Shaw 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 CCT motif family subclass of the CCT gene family with only a single CCT domain (Cockram et al., 2012). *Ghd7* is up-regulated under LD conditions and represses *Hd3a* and flowering time. *VERNALIZATION2* (*VRN-H2*), a barley homolog of the LD repressor *Ghd7* in rice, is also up-regulated 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 FLOWERING3* (*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 (Fujino and Sekiguchi, 2005; Shibaya et al., 2011). 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 contributed significantly 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 with the wild-type *Ppd-H1* allele, but it is not associated with flowering variation under SDs (Laurie et al., 1995; Decousset et al., 2000; Turner et al., 2005; Hemming et al., 2008). 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 down-regulated during vernalization by *VRN-H1*, an *APETALA1/FRUITFUL*-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 the 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 *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* up-regulated the expression of *VRN-H2*, which was associated with a delay in flowering under LD and SD conditions as compared with 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 the 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 the expression of major flowering time genes under LDs and SDs.

Under LDs, transgenic *Ubi::HvCO2* lines flowered on average 36 d after emergence (DAE) and thus significantly earlier than the null segregants and wild-type Golden Promise, which required on average 54 d to flower (Fig. 1). Under SD conditions, overexpression of *HvCO2* induced flowering, whereas the null segregants and the wild type 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 daylength-dependent effects of *Ubi::HvCO2* on flowering time, we evaluated the expression of *HvCO2*, *HvCO1*, and 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 up-regulated in the transgenic lines compared with the null segregants and the wild type under LD and SD conditions (Fig. 2A). Expression of *HvCO1* was significantly reduced in all *Ubi::HvCO2* lines compared with the null segregants and the wild type under LDs. Under SDs, the expression of *HvCO1* was below the detection limit at the time when the seedlings were sampled (Fig. 2B).

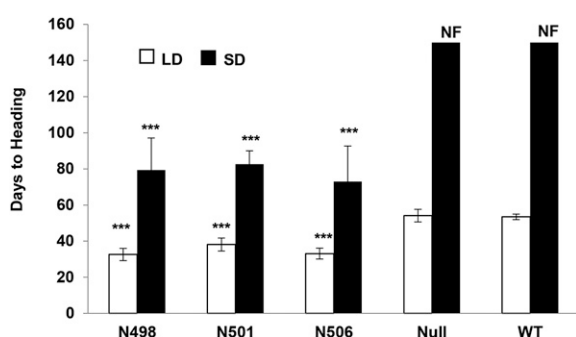The expression of *HvFT1* was significantly up-regulated in all tested transgenic lines under LDs but was below the detection level in the null segregants and the wild type (Fig. 2C). Under SD conditions, however, the expression of *HvFT1* was not detected in any of the tested genotypes. The expression of *HvFT3* was not different between transgenic and nontransgenic plants under LDs but was down-regulated in all four *Ubi::HvCO2* lines as compared with the wild type and the null segregants under SDs (Fig. 2D). In addition, overexpression of *HvCO2* caused a significant up-regulation of *VRN-H1* under LDs, whereas differences in *VRN-H1* expression between *Ubi::HvCO2* lines, the wild type, and the null segregants were not consistent under SDs (Fig. 2E). Expression levels of *Ppd-H1* in the *Ubi::HvCO2* lines did not differ significantly from those in nontransgenic controls under LDs and SDs (Fig. 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

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 F2 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 *VRN-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 the overexpression of *HvCO2* can overcome the vernalization requirement, F2 plants were grown without vernalization under LDs and scored for flowering time.

Flowering time varied between 23 and 130 d in the F2 population of *Ubi::HvCO2* × Igri under LDs (Supplemental Fig. S1). The F2 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 d.

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 F2 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

Figure 1. Analysis of the flowering time of *Ubi::HvCO2* transgenic lines under LD and SD conditions. Flowering time is shown for *Ubi::HvCO2* transgenic lines (N498, N501, and N506), the null segregant line (Null), and Golden Promise (WT) grown under LD (white bars; 16 h of light) and SD (black bars; 8 h of light) conditions. Flowering time was measured for five to 20 plants for each of the *Ubi::HvCO2* lines, the null segregant line, and the wild type in days from germination until heading. Null and wild-type plants did not flower at the end of the experiment (NF; 150 d) under SDs. Columns represent the average flowering time. Error bars indicate SD. Asterisks refer to significant differences in flowering time of the transgenic lines compared with the null and wild-type plants (***, $P < 0.001$).
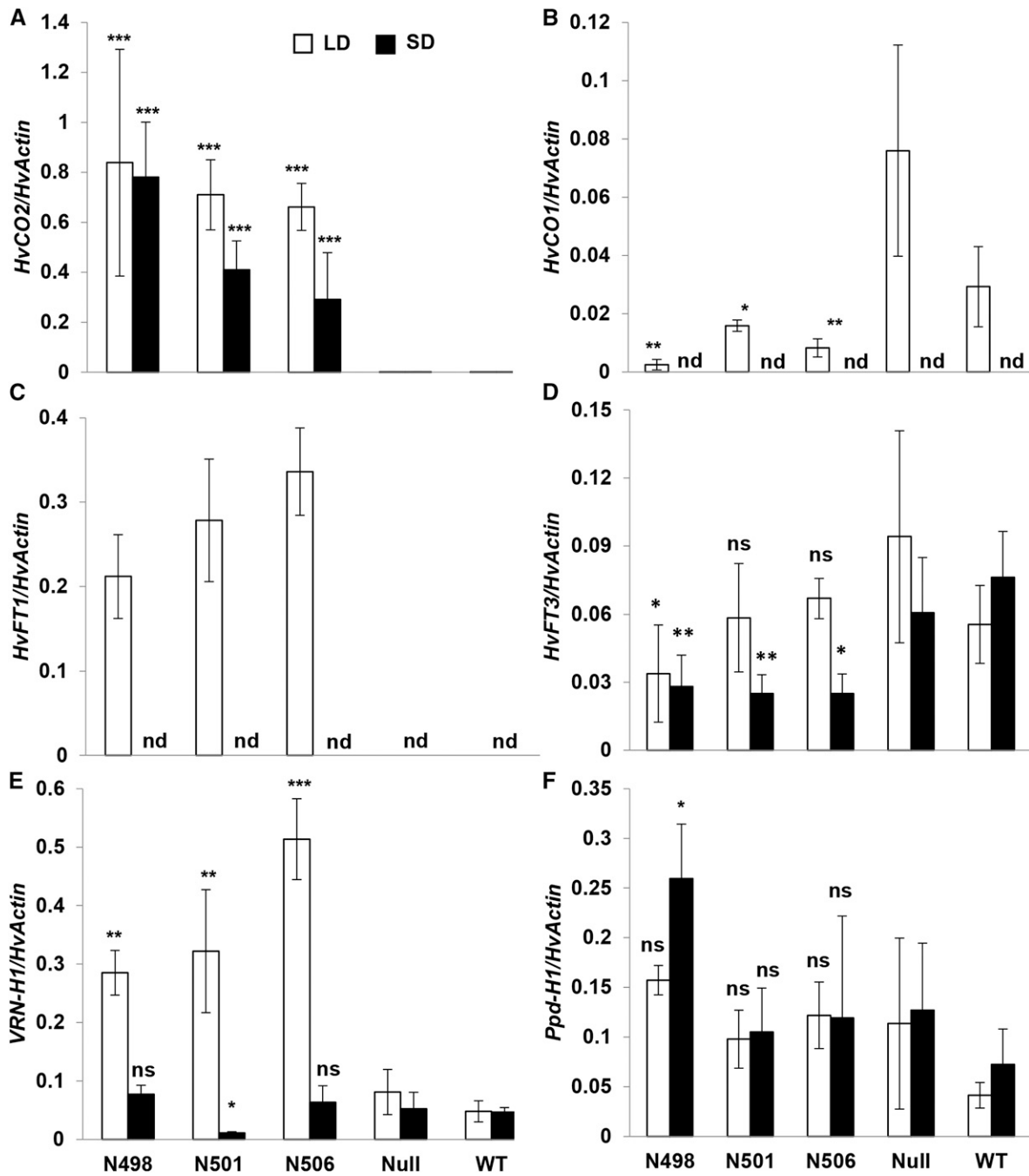
Figure 2. Expression levels of flowering time genes in *Ubi::HvCO2* transgenic lines. Expression levels of flowering time genes are shown for *Ubi::HvCO2* transgenic lines (N498, N501, and N506), null segregants (Null), and Golden Promise (WT) under LD (white bars; 16 h of light) and SD (black bars; 8 h of light) conditions. Expression analysis was performed on leaf samples collected 2 h before the end of the light period at day 7 after germination under LDs and SDs. For each transgenic line, the null segregant line, and the wild type, three to seven plants were used as biological replicates. Columns represent the average expression of *HvCO2* (A), *HvCO1* (B), *HvFT1* (C), *HvFT3* (D), *VRN-H1* (E), and *Ppd-H1* (F), all normalized to the expression level of *HvACTIN*. nd, No expression detected. Error bars indicate SD. Asterisks refer to significant expression differences in the transgenic lines compared with the null and wild-type plants (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$). ns, No significant difference in expression at $P < 0.05$. Statistical comparisons were performed separately for gene expression measured under LDs and SDs.

with flowering time in the spring/facultative F2 subpopulation comprising all F2 genotypes with a deletion of the *VRN-H2* locus. We designated alleles segregating in the F2 population and derived from the winter parent with W (winter) and alleles derived from the spring barley Golden Promise with S (spring).

In total, the overexpression of *HvCO2* and allelic variation at *VRN-H1*, *VRN-H2*, and *Ppd-H1* accounted for 89% of the variation identified for flowering time in the F2 population grown under LDs (Supplemental Table S1). Natural variation at *HvFT3* did not show any significant effect on flowering time under LDs. The transgene *Ubi::HvCO2* accelerated flowering time but explained only 16% of the overall phenotypic variation (Fig. 3A; Supplemental Table S1). In contrast, natural variation at *VRN-H2* had the strongest effect on flowering time and accounted for 51% of the flowering time variation (Fig. 3B; Supplemental Table S1). F2 genotypes carrying the winter allele of *VRN-H2* flowered on average after 74 d and thus 42 d later than those carrying the deletion (spring allele) of the gene. The vernalization gene *VRN-H1* explained 11% of the variation in days to flowering, as the winter allele delayed flowering time by an average of 18 d. Furthermore, the interaction between *VRN-H1* and *VRN-H2* was significant and explained 3% of the phenotypic variation. The combination of winter alleles at *VRN-H2* and *VRN-H1* delayed flowering time by an additional 22 d compared with the sum of the effects of the winter alleles at both genes. The vernalization genes *VRN-H1* and *VRN-H2* and their interaction thus explained in total 65% of flowering time variation in the population. Consequently, the effects of *VRN-H2* and *VRN-H1* had more pronounced effects on time to flowering than *Ubi::HvCO2*. Nevertheless, *Ubi::HvCO2* reduced days to heading in the winter F2 plants with homozygous and heterozygous winter alleles at *VRN-H1* and *VRN-H2*, respectively, by about 22 d (Supplemental Fig. S2A). Allelic variation at the major photoperiod gene *Ppd-H1* explained 5% of the overall variation in days to flowering. The photoperiod-responsive allele reduced time to flowering by 8 d compared with the mutated *ppd-H1* allele. In spring or facultative F2 genotypes with a deletion of *VRN-H2*, *Ppd-H1* exerted the strongest effect on flowering time (65%; Supplemental Table S2) even in the presence of the transgene (Supplemental Fig. S2B). The wild-type *Ppd-H1* allele accelerated flowering time by 11 d as compared with the mutated *ppd-H1* allele in the transgenic F2 genotypes with a deletion of *VRN-H2*.

Taken together, the repressive effect of *VRN-H2* was stronger than the effect of *Ubi::HvCO2* on flowering.

Nevertheless, the presence of the transgene accelerated flowering time also in the winter genotypes. In transgenic F2 genotypes with a deletion of the *VRN-H2* locus, variation at *Ppd-H1* had the strongest effect on flowering time, consistent with the observation that transgenic genotypes maintained a strong photoperiod response.

Overexpression of *HvCO2* Up-Regulated the Floral Repressor *VRN-H2*

To further characterize the molecular control of flowering time in the F2 population, we analyzed the effects of *Ubi::HvCO2* on the expression levels of selected flowering time regulators.

HvCO2 expression in F2 genotypes carrying the transgene was on average 1,000 times higher than in the nontransgenic F2 genotypes (Fig. 4A). Accordingly, the presence/absence of the transgene explained 72% of the variation in *HvCO2* expression (Supplemental Table S3). Interestingly, the presence of *VRN-H2* was associated with a significant down-regulation of *HvCO2* expression in F2 genotypes carrying the wild-type *HvCO2* gene (Supplemental Fig. S3). *HvCO2* expression levels showed a high negative correlation with days to flowering (-0.58; Supplemental Table S4). In addition, *HvCO2* exhibited a high positive correlation with the expression levels of *Ppd-H1* (0.60) in the winter F2 population but not in the spring F2 population (Supplemental 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 (Supplemental Table S3). Interestingly, winter F2 genotypes carrying the *Ubi::HvCO2* transgene showed on average an 11 times higher expression of *VRN-H2* than F2 genotypes without the transgene (Fig. 4B). Accordingly, the expression levels of *HvCO2* and *VRN-H2* were highly correlated (0.79) in winter F2 genotypes (Supplemental Table S5). Despite the strong up-regulation of the flowering repressor *VRN-H2* in the presence of *Ubi::HvCO2*, transgenic winter F2 genotypes flowered earlier than the nontransgenic winter F2 genotypes (Supplemental Fig. S2A). In addition,
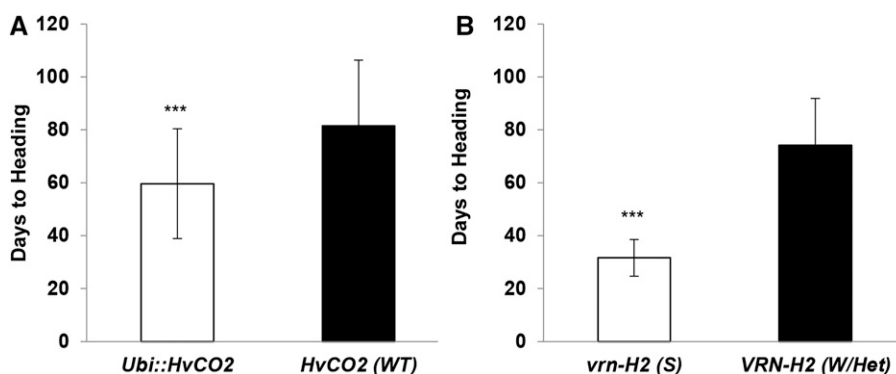

Figure 3. Effects of *Ubi::HvCO2* and *VRN-H2* on flowering time of the F2 population *Ubi::HvCO2* × Igri under LD conditions. Columns represent the average flowering time of F2 genotypes classified according to the presence/absence of the transgene *Ubi::HvCO2* (A) and the allelic variation of *VRN-H2* (B). S, Spring allele; W/Het, homozygous and heterozygous winter allele; WT, wild type. Error bars indicate SD. Asterisks refer to a significant difference (***, $P < 0.001$).
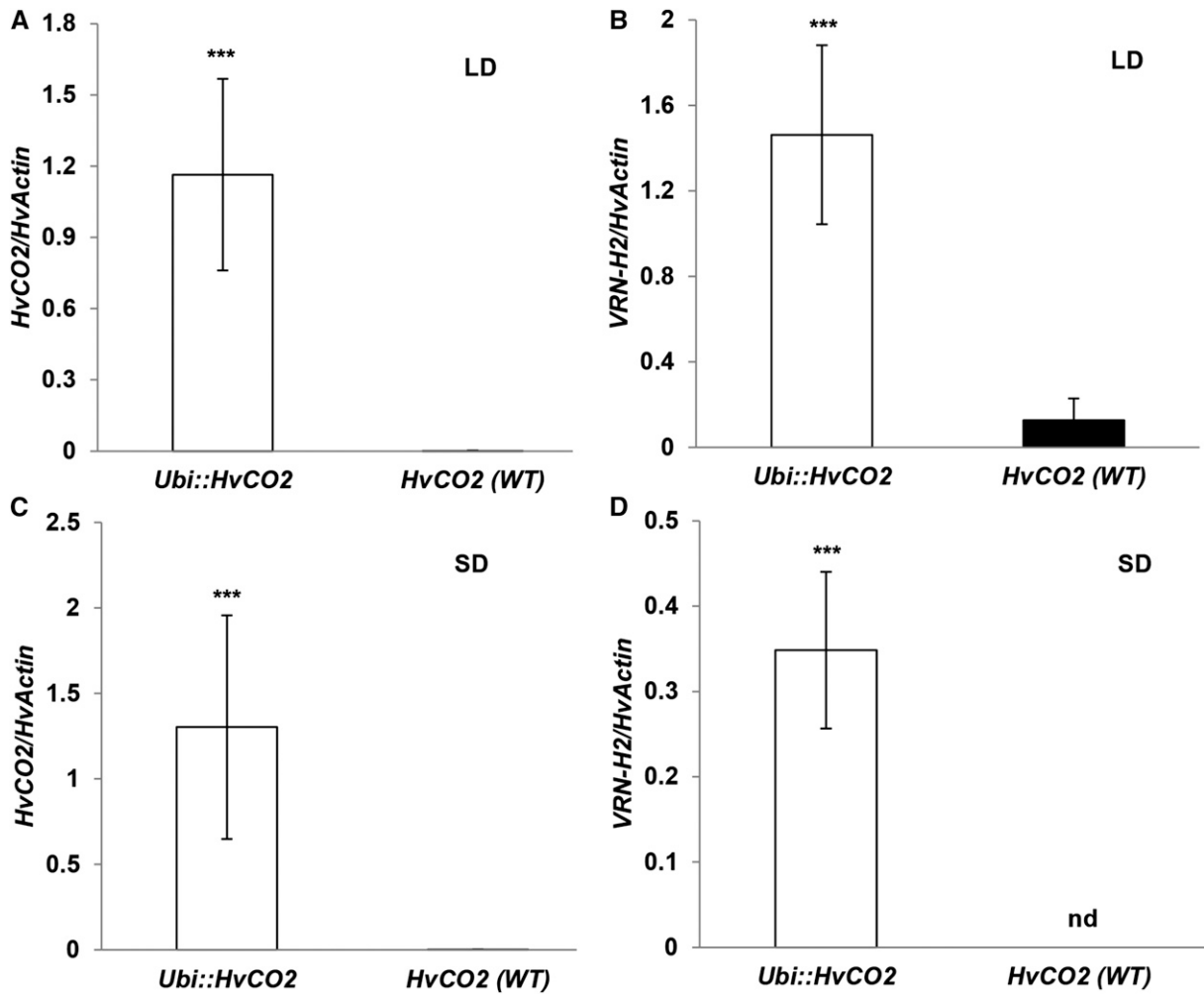
Figure 4. Effects of *Ubi*::*HvCO2* on the expression of *HvCO2* and *VRN-H2* in F2 genotypes of the population *Ubi*::*HvCO2* × Igri grown under LD and SD conditions. Columns represent the average expression of *HvCO2* (A and C) and *VRN-H2* (B and D), each normalized to *HvACTIN* in F2 genotypes classified according to the presence/absence of the transgene *Ubi*::*HvCO2* under LD (16 h of light; A and B) and SD (8 h of light; C and D) conditions. F2 genotypes with the deleted *VRN-H2* locus were not considered in B and D. Expression analysis was performed on leaf samples collected 2 h before the end of the light period at day 7 after germination. nd, No expression detected; WT, wild type. Error bars indicate SD. Asterisks refer to significant differences (***, $P < 0.001$).

VRN-H2 was significantly up-regulated by the wild-type allele of *Ppd-H1* in the background of nontransgenic F2 genotypes (Fig. 5A). The presence of *VRN-H2*, in turn, correlated with the down-regulation of *Ppd-H1*, in particular in the background of the nontransgenic genotypes (Fig. 5B).

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 F2 genotypes independent of the transgene (Supplemental Fig. S4). On the other hand, F2 genotypes with the *Ubi*::*HvCO2* transgene showed higher expression levels of *HvFT1* in the F2 genotypes with a deletion of the *VRN-H2* locus (Fig. 6B). Accordingly, across the entire population, variation in *HvFT1* expression was mainly controlled by *VRN-H2* (35%) and *Ubi*::*HvCO2* (22%;

Supplemental Table S3). Interestingly, the photoperiod-responsive allele of *Ppd-H1* significantly up-regulated the expression of *HvFT1* in the spring/facultative F2 genotypes with a deletion of *VRN-H2* in the presence of the transgene *Ubi*::*HvCO2* (Supplemental Fig. S5). *HvFT1* expression levels strongly correlated with days to flowering (−0.70) and with the expression levels of *VRN-H1* (0.57), *Ppd-H1* (0.33), and *VRN-H2* (−0.47; Supplemental Table S4).

In summary, *Ubi*::*HvCO2* caused a strong up-regulation of *VRN-H2*. In addition, variation at *Ppd-H1* affected *VRN-H2* expression in the nontransgenic F2 subpopulation. *VRN-H2*, in turn, was involved in the down-regulation of *Ppd-H1* and *HvCO2*. Our findings thus suggest strong reciprocal interactions between *HvCO2*, *Ppd-H1*, and *VRN-H2*. *Ubi*::*HvCO2* and *Ppd-H1*
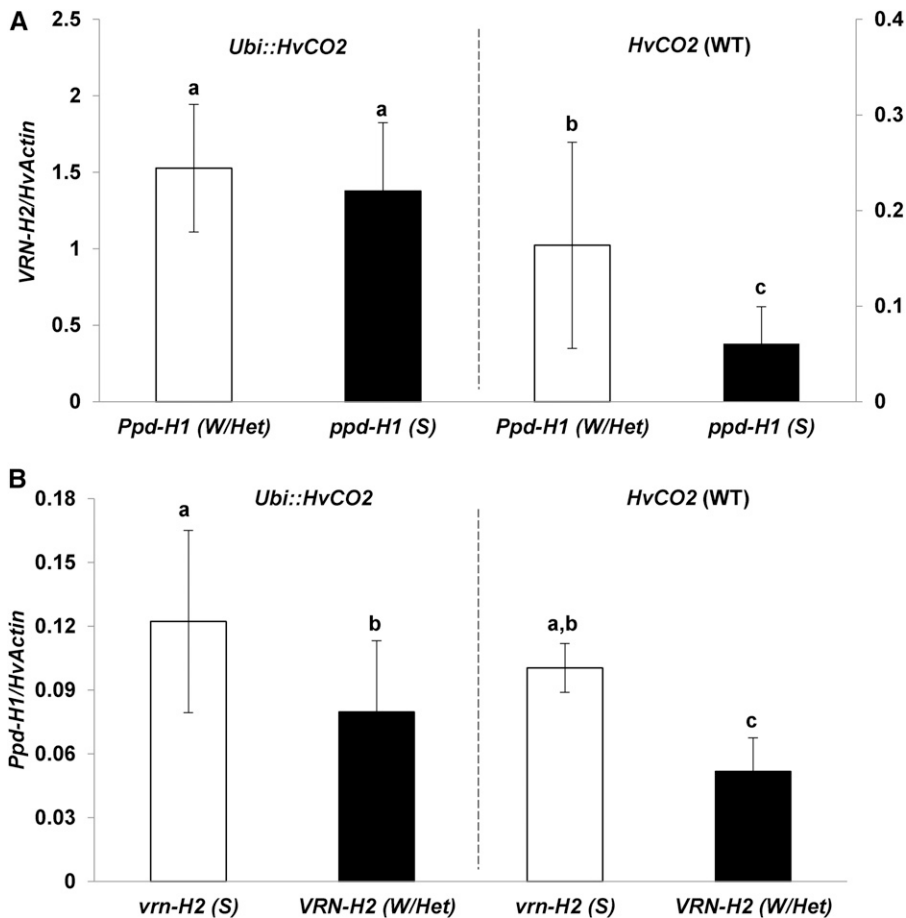
Figure 5. Reciprocal interaction between *Ppd-H1* and *VRN-H2* in the F2 population *Ubi::HvCO2* × Igri under LD conditions. A, Columns represent the average expression of *VRN-H2* normalized to *HvACTIN* in F2 genotypes classified according to the presence/absence of *Ubi::HvCO2* and allelic variation at *Ppd-H1*. F2 genotypes with the deleted *VRN-H2* locus were not considered. B, Columns represent the average expression of *Ppd-H1* normalized to *HvACTIN* in F2 genotypes classified according to the presence/absence of *Ubi::HvCO2* and *VRN-H2*. S, Spring allele; W/Het, homozygous and heterozygous winter allele; WT, wild type. Expression analysis was performed on leaf samples collected 2 h before the end of the light period in LDs (16 h of light) at day 7 after germination. Error bars indicate SD. Letters on top of each column indicate significant differences in expression levels at $P < 0.05$.

exhibited additive effects on *HvFT1* expression in the absence of *VRN-H2*.

Overexpression of *HvCO2* and *HvCO1* Induced the Expression of *VRN-H2* and *HvFT1* under SD Conditions

As the overexpression of *HvCO2* up-regulated the expression of *VRN-H2* under LDs, we further tested if *Ubi::HvCO2* also up-regulated *VRN-H2* expression under SDs, when the gene is usually not expressed. For this purpose, 168 F2 genotypes derived from the cross *Ubi::HvCO2* × Igri were grown in the greenhouse under SD conditions (8–10 h of light) and scored for flowering time and gene expression.

Overexpression of *HvCO2* caused an up-regulation of *VRN-H2* in the transgenic F2 genotypes also under SDs at 7 DAE, while no *VRN-H2* expression was detected in the nontransgenic F2 genotypes (Fig. 4, C and D). Transgenic F2 genotypes with the *VRN-H2* locus flowered on average after 125 DAE, while transgenic F2 genotypes with a deletion of *VRN-H2* required on average 74 d to flower under SDs (Fig. 6A). All nontransgenic F2 genotypes failed to flower up to 200 DAE (when the experiment was stopped). The 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 (Supplemental Table S6). Expression levels of *HvFT1* were under the detection limit at 7 DAE but were later (75 DAE) detected in transgenic F2 genotypes under SDs (Fig. 6B). Transgenic F2 genotypes with a deletion of *VRN-H2* had 6-fold increased expression levels of *HvFT1* as compared with their siblings with the winter allele of *VRN-H2*. Expression of *HvFT1* was not detected in the nontransgenic F2 genotypes. Variation in *HvFT1* expression was thus mainly controlled by *VRN-H2* (29%) and *Ubi::HvCO2* (16%; Supplemental Table S6). Finally, variation at *Ppd-H1* affected flowering time and *HvFT1* expression in the transgenic F2 genotypes under SDs, when *Ppd-H1* does usually not have any effect on time to flowering (Supplemental Table S6).

We further tested if overexpression of *HvCO1*, as the closest homolog of *HvCO2*, could also influence *VRN-H2* expression under LD and noninductive SD conditions. The up-regulation of *HvCO1* expression in *Ubi::HvCO1* × Igri F2 genotypes carrying the *Ubi::HvCO1* transgene was associated with an up-regulation of *VRN-H2* under LDs and SDs (Supplemental Fig. S6).

Taken together, *Ubi::HvCO1* and *Ubi::HvCO2* up-regulated the expression of *VRN-H2* under LDs and SDs. Up-regulation 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 with *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 wild-type, F2 genotypes under SDs.

DISCUSSION

Overexpression of *HvCO2* Causes Photoperiod-Dependent Early Flowering in Barley

In Arabidopsis, *CO* is an important promoter of flowering in response to LDs (Koornneef et al., 1991; Putterill et al., 1995). Arabidopsis plants constitutively overexpressing *CO* were early flowering and almost completely insensitive to daylength (Onouchi et al., 2000). In our study, overexpression of *HvCO2*, which represents 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 daylength and flowered significantly earlier under LDs than under SDs. Accordingly, *HvFT1* up-regulation in the transgenic lines occurred significantly later under SDs when compared with LD conditions. Analysis of flowering time and gene expression in the cross *Ubi::HvCO2* × Igri suggested that the photoperiod response of transgenic lines was influenced by *Ppd-H1*. Variation at *Ppd-H1* affected flowering time and the expression of *HvFT1* in transgenic spring F2 genotypes under LDs. Consequently, *Ppd-H1* controlled flowering time downstream of *HvCO2* expression under LDs (Fig. 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 daylength and natural variation at *Ppd-H1*. In contrast, up-regulation of *Ppd-H1* expression in a barley mutant with a nonfunctional *HvELF3* gene was associated with photoperiod-independent early flowering (Faure et al.,

Figure 6. Effects of *Ubi::HvCO2* and allelic variation of *VRN-H2* on flowering time and *HvFT1* expression in *Ubi::HvCO2* × Igri F2 genotypes under SD conditions. The F2 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; WT, wild type. A, Columns represent the average flowering time of the different genotypic classes. Nontransgenic F2 genotypes did not flower at the end of the experiment (NF; 200 d). 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 2 h before the end of the light period from a subset of F2 genotypes that did not flower by the date of sampling (75 DAE). nd, No expression detected. Error bars indicate SD. Asterisks refer to significant differences (***, $P < 0.001$).
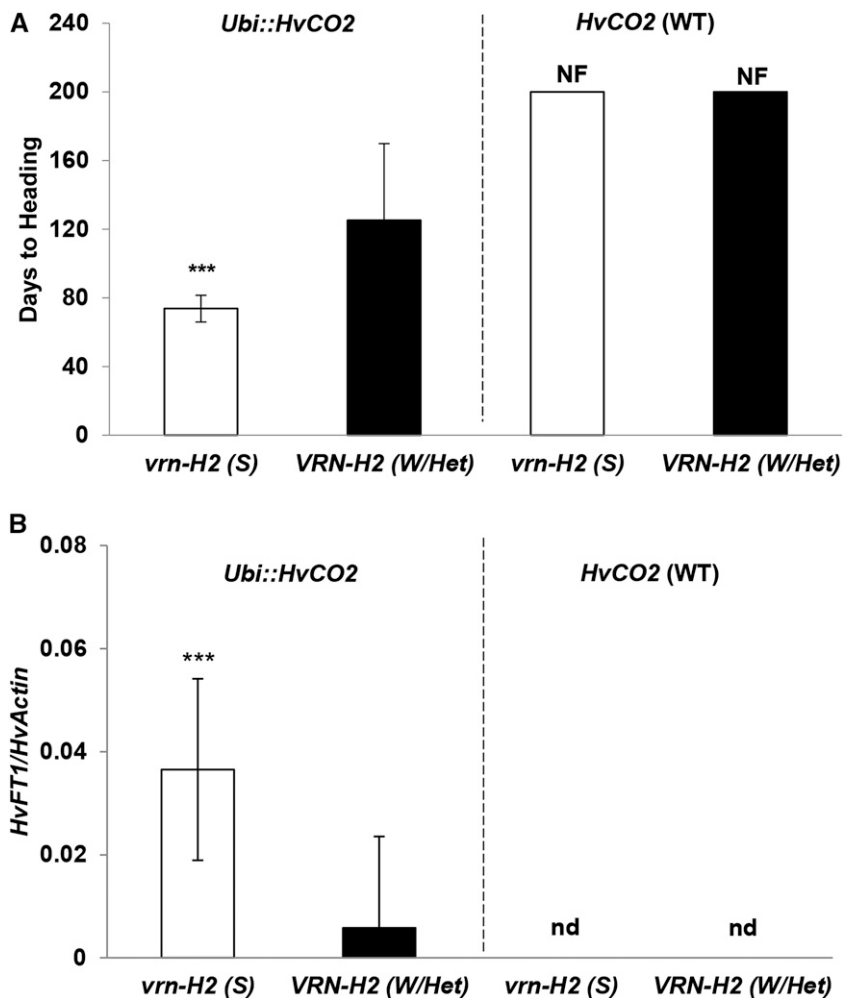
2012). Moreover, natural mutations in the promoters of the homologous *PPD1* genes in wheat and consequent up-regulation of *PPD1* expression caused photoperiod insensitivity and early flowering (Beales et al., 2007). These reports, together with our results, indicate that the expression variation of *Ppd1* has a stronger impact on photoperiodic flowering than expression changes of *CO1/CO2*. Similarly, a rice line with a nonfunctional allele at *Hd1* (rice *CO*) retained sensitivity to daylength, and complete daylength insensitivity was only observed when alleles at both *Hd1* (rice *CO*) and *Hd2* (*OsPRR37*) were nonfunctional (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 (*Sorghum bicolor*) 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 the 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; 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 stabilize the 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 nonfunctional alleles at *HvCO1* and *HvCO2* would further help to dissect the genetic interactions of *Ppd-H1* and *HvCO1/CO2* in barley.
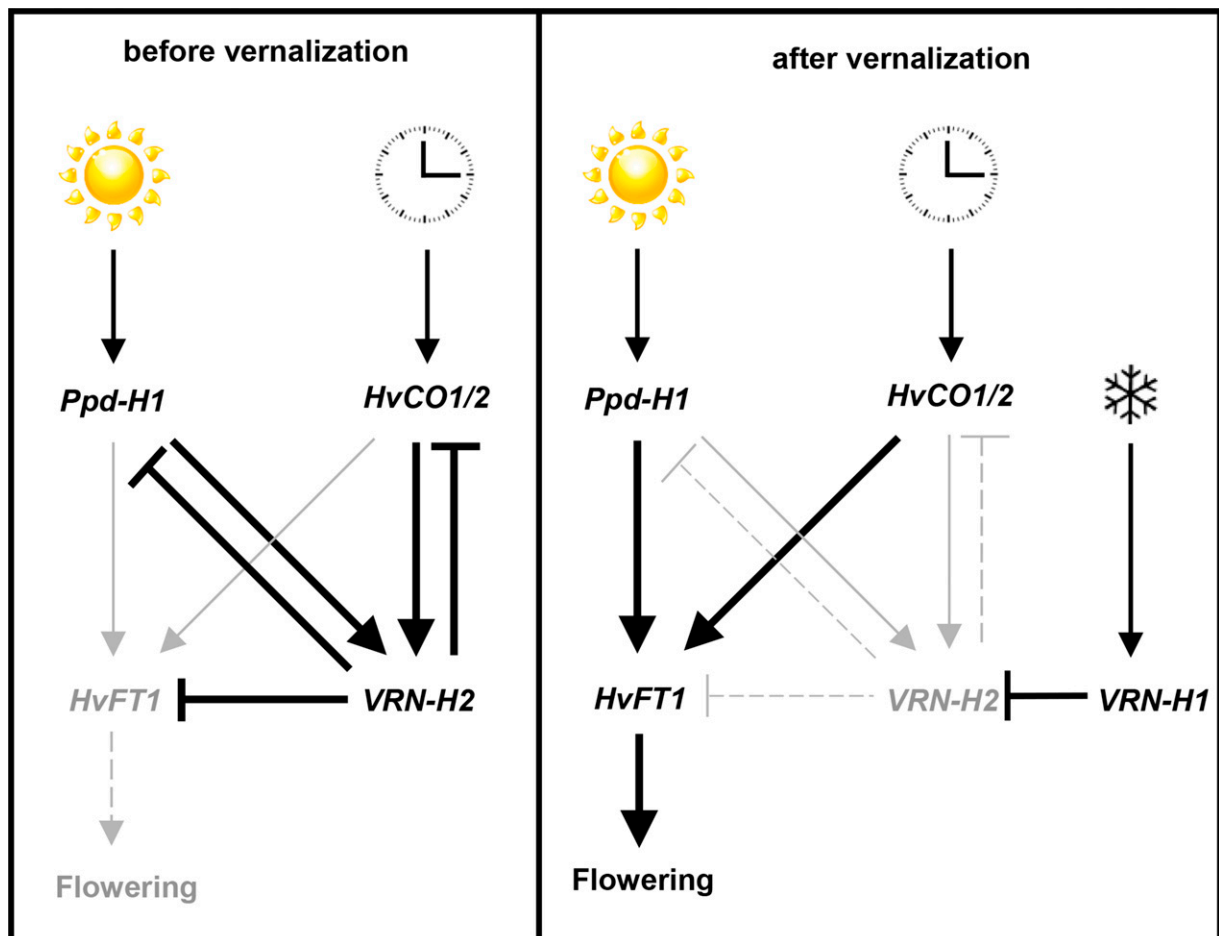

Figure 7. Model for the coregulation of *VRN-H2* and *HvFT1* by *HvCO1/CO2* and *Ppd-H1* in winter barley under LD conditions before and after vernalization. *HvCO1/CO2* and *Ppd-H1* induce the expression of *VRN-H2*, which acts as a strong repressor of *HvFT1* and flowering time in winter barley before vernalization. Up-regulation 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* up-regulate *HvFT1* and induce flowering under LD conditions.

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 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 up-regulation 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 ortholog of *CO*, was also proposed to have these two opposite functions of repressing and promoting flowering by inhibiting and inducing *Hd3a* expression (*FT* ortholog) under LDs and SDs, respectively (Yano et al., 2000; Hayama et al., 2003). Consequently, the involvement of *CO* in the 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 that, like *Vrn-H2*, is up-regulated under LDs and represses the expression of *Hd3a* (Xue et al., 2008). It was suggested that *Ghd7* and *Hd1* independently control the photoperiod response in rice (Xue et al., 2008; Tsuji et al., 2011). However, Saito et al. (2012) showed 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 F2 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, exhibited up-regulated expression levels of *VRN-H2* under noninductive SDs (Turner et al., 2013). Moreover, expression studies in wheat *phyC* mutants revealed a correlated down-regulation of *PPD1* and *VRN-H2* (Chen et al., 2014). These findings indicate that *Ppd-H1* is involved in the regulation of *VRN-H2*. Thus, *Ppd-H1* may also act as an indirect repressor of flowering by up-regulating *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* (Murphy et al., 2011; Koo et al., 2013).

Our results showed that functional allelic diversity at *Ppd-H1* and overexpression of *HvCO1/CO2* could influence *VRN-H2* expression (Fig. 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 up-regulation of *Ppd-H1* under LDs, indicating that both genes may also have interacted transcriptionally. Furthermore, the 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, the 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; Yan et al., 2004; Turner et al., 2005; 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 Heme Activator Protein (HAP)/Nuclear Factor Y (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 stress (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 the expression levels of *HvFT1* and daylength sensitivity were controlled by *Ppd-H1* downstream of *HvCO2* overexpression (Fig. 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 activate flowering after vernalization when *VRN-H2* is down-regulated. Consequently, floral repression through *VRN-H2* and floral activation through *HvFT1* are 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 also affects the vernalization response.

MATERIALS AND METHODS

Generation of Transgenic *Ubi::HvCO2* Lines and Their Growth Conditions

Barley (*Hordeum vulgare*) plants of the spring variety Golden Promise were transformed with an overexpression construct generated with the complementary DNA (cDNA) clones of *HvCO2* (AF490470) driven by the maize (*Zea mays*) 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*. *A. tumefaciens*-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 (Supplemental Table S7). The generation of transgenic *Ubi::HvCO1* lines is described by Campoli et al. (2012).

Three independent transgenic T2 families, designated *Ubi::HvCO2* lines N498, N501, and N506, a null segregant control line that lost the transgene, and the wild-type Golden Promise were sown in soil and grown under LD (16 of light/8 h of dark) and SD (8 h of light/16 h of dark) conditions in the greenhouse (temperature, 20°C/16°C days/nights). Five to 20 plants of the transgenic line, the null segregants, and the wild type were used to score flowering time, which was measured in days from emergence until heading (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 2 h before the end of the light period under LDs and SDs (ZT14 [for Zeitgeber time] under LDs and ZT6 under SDs).

Generation of *Ubi::HvCO2* × Igri and *Ubi::HvCO1* × Igri F2 Populations and Their Growth Conditions

For generation of the F2 populations, each of the transgenic lines *Ubi::HvCO2* N506 and *Ubi::HvCO1* N2330 was crossed with the winter barley Igri. Wild-type Golden Promise, 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 wild type 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 that 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 F2 populations, alleles derived from the winter parent Igri are designated with W and alleles derived from the spring parent Golden Promise are designated with S.

One hundred ninety-one F2 plants and 168 F2 plants derived from the cross *Ubi::HvCO2* × Igri were sown in soil and grown in the greenhouse (temperature, 20°C/16°C days/nights) under LD (16 h of light/8 h of dark) and SD (8 h of light/16 h of dark) conditions, respectively. After 50 d in 8-h SD conditions, the light period was extended to 10 h 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 F2 genotypes 7 DAE at ZT14 under LDs and from all 168 F2 genotypes 7 DAE at ZT6 under SDs and subsequently used for RNA extraction and gene expression analysis. The selection of F2 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 F2 genotypes grown under SDs 75 DAE (25 d after extending the photoperiod to 10 h). Selection of the genotypes was also based on genotypic information and excluded genotypes that had already flowered by the time of sampling.

Similarly, 80 F2 genotypes derived from the cross *Ubi::HvCO1* × Igri were grown under the same LD and SD conditions. The F2 genotypes were genotyped for the transgene *Ubi::HvCO1* and *VRN-H2*. Expression analysis was performed on a subset of 20 F2 genotypes under LDs and 12 F2 genotypes under SDs, which had been selected for the dominant winter allele *VRN-H2* but segregated for the presence of the transgene. The 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 F2 genotypes was extracted from leaf samples following the Biosprint DNA extraction protocol (Qiagen). F2 genotypes of all analyzed populations were genotyped for the presence of the transgene and the 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). PCR was performed as described in the original references (primers are listed in Supplemental Table S7).

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA extraction, first-strand cDNA synthesis, and quantitative real-time PCR for individual F2 plants were performed as described by Campoli et al. (2012). Quantitative real-time PCR was performed using gene-specific primers (Supplemental 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 gene (*HvACTIN*) using the LightCycler 480 Software (version 1.5; Roche).

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 (wild type + null combined) grown under LDs and SDs was determined using Student's *t* test. A fixed-model 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 F2 populations. Pearson correlation coefficients were calculated between flowering time and gene expression values in the tested populations.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Flowering time of the F2 population *Ubi::HvCO2* × Igri under LDs.

Supplemental Figure S2. Effects of *Ubi::HvCO2* and *Ppd-H1* on flowering time of different F2 subpopulations of *Ubi::HvCO2* × Igri under LDs.

Supplemental Figure S3. Effects of *VRN-H2* on expression of *HvCO2* in the F2 population *Ubi::HvCO2* × Igri under LDs.

Supplemental Figure S4. Effects of *Ubi::HvCO2* and *VRN-H2* on expression of *HvFT1* in the F2 population *Ubi::HvCO2* × Igri under LDs.

Supplemental Figure S5. Effect of *Ppd-H1* on expression levels of *HvFT1* in the transgenic spring/facultative F2 subpopulation under LDs.

Supplemental Figure S6. Effects of *Ubi::HvCO1* on expression of *HvCO1* and *VRN-H2* in the F2 population *Ubi::HvCO1* × Igri under LDs and SDs.

Supplemental Table S1. Analysis of variance (ANOVA) of flowering time of the F2 population *Ubi::HvCO2* × Igri under LDs.

Supplemental Table S2. ANOVA of flowering time of the spring/facultative subpopulation *Ubi::HvCO2* × Igri F2 population under LDs.

Supplemental Table S3. ANOVA for expression of flowering time genes in the F₂ population Ubi::HvCO2 × Igri LDs.

Supplemental Table S4. Pearson correlation coefficients of flowering time and expression of flowering genes in the F$_2$ population Ubi::HvCO2 × Igri under LDs.

Supplemental Table S5. Pearson correlation coefficients of flowering time and expression of flowering genes in the spring/facultative and winter subpopulations of the F$_2$ population Ubi::HvCO2 × Igri under LDs.

Supplemental Table S6. ANOVA of HvFT1 expression and flowering time of the F$_2$ population Ubi::HvCO2 × Igri under SDs.

Supplemental Table S7. List of primers used in this study.

ACKNOWLEDGMENTS

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