Low-Temperature and Daylength Cues Are Integrated to Regulate FLOWERING LOCUS T in Barley†[C][W][OA]

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Interactions between flowering time genes were examined in a doubled haploid barley (Hordeum vulgare) population segregating for H. vulgare VERNALIZATION1 (HvVRN1), HvVRN2, and PHOTOPERIOD1 (PPD-H1). A deletion allele of HvVRN2 was associated with rapid inflorescence initiation and early flowering, but only in lines with an active allele of PPD-H1. In these lines, the floral promoter FLOWERING LOCUS T (HvFT1) was expressed at high levels without vernalization, and this preceded induction of HvVRN1. Lines with the deletion allele of HvVRN2 and the inactive ppd-H1 allele did not undergo rapid inflorescence initiation and were late flowering. These data suggest that HvVRN2 counteracts PPD-H1 to prevent flowering prior to vernalization. An allele of HvVRN1 that is expressed at high basal levels (HvVRN1-I) was associated with rapid inflorescence initiation regardless of HvVRN2 or PPD-H1 genotype. HvFT1 was expressed without vernalization in lines with the HvVRN1-I allele and HvFT1 transcript levels were highest in lines with the active PPD-H1 allele; this correlated with rapid apex development postinflorescence initiation. Thus, expression of HvVRN1 promotes inflorescence initiation and up-regulates HvFT1. Analysis of HvVRN1 expression in different genetic backgrounds postvernalization showed that HvVRN2, HvFT1, and PPD-H1 are unlikely to play a role in low-temperature induction of HvVRN1. In a vernalization responsive barley, HvFT1 is not induced by low temperatures alone, but can be induced by long days following prolonged low-temperature treatment. We conclude that low-temperature and daylength flowering-response pathways are integrated to control expression of HvFT1 in barley, and that this might occur through regulation of HvVRN2 activity.

Flowering of temperate cereals, such as bread wheat (Triticum aestivum) and barley (Hordeum vulgare), is accelerated by prolonged exposure to cold, or vernalization. Vernalization occurs during winter when plants are exposed to temperatures between zero and 10°C (Takahashi and Yasuda, 1971; Flood and Halloran, 1984). Vernalization accelerates flowering by promoting inflorescence initiation, the first step in the transition of the shoot apex to reproductive development (Flood and Halloran, 1984). Temperate cereal varieties differ in their requirement for vernalization. Some varieties have an obligate requirement for vernalization, whereas others flower early without vernalization. Three genes have been reported to determine the requirement for vernalization in the temperate cereals: VERNALIZATION1 (VRN1), VRN2, and VRN3 (Pugsley, 1970; Takahashi and Yasuda, 1971; Dubcovsky et al., 1998). Gene sequences for these three loci have been identified (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003, 2004, 2006).

VRN1 encodes a MADS-box transcription factor that is essential for flowering (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Shizukawa et al., 2007). In varieties that require vernalization to flower, VRN1 is expressed at low basal levels and is induced by exposure to low temperatures (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). Some varieties of wheat and barley carry alleles of VRN1 that are expressed at high basal levels without vernalization. These alleles of VRN1 reduce or remove the requirement for vernalization and are dominant to alleles that are expressed at low basal levels without vernalization (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). In barley, dominant alleles of H. vulgare VRN1 (HvVRN1) have deletions of regions in the first intron, which are presumably essential for maintaining low levels of HvVRN1 expression in plants that have not been vernalized (Fu et al., 2005).

VRN2 encodes a protein with a zinc finger motif and a CCT (CONSTANS, CONSTANS-LIKE, and TIMING OF CAB1-1) domain (Yan et al., 2004). VRN2 is a repressor of flowering and plants that lack a functional copy of VRN2 flower early (Yan et al., 2004). When plants are vernalized in long days, expression of VRN2 decreases, whereas expression of VRN1 increases (Yan et al., 2004). On the basis of this expression pattern, it was suggested that VRN2 represses VRN1, and that vernalization represses VRN2, permitting induction of
eties that require vernalization, \( \text{PHOTOPERIOD} \) mediate the long-day flowering response (Turner et al., 1999; Yan et al., 2006; Faure et al., 2007). FT is a mobile promoter of flowering; FT protein is produced in leaves when Arabidopsis plants are exposed to long days and is transported to the shoot apex where it promotes floral development (Corbesier et al., 2007). Similarly, in barley is expressed at elevated levels without cold treatment (Yan et al., 2006). The association between dominant alleles of VRN3, which cause early flowering and remove the requirement for vernalization, \( \text{HvFT1} \) is expressed at elevated levels without cold treatment (Yan et al., 2006). The association between dominant alleles of VRN3 and elevated \( \text{HvFT1} \) expression levels, combined with tight genetic linkage, suggest that activation of \( \text{HvFT1} \) might be the molecular basis for dominant alleles of VRN3 (Yan et al., 2006). Mutations in the first intron of \( \text{HvFT1} \) might be the basis for these high expression levels (Yan et al., 2006). It is not clear how daylength and low-temperature pathways are integrated to regulate \( \text{HvFT1} \) expression.

In this article we show that loss of \( \text{HoVRN2} \) allows rapid inflorescence initiation only when an active allele of \( \text{PPD-H1} \) is present, and this is correlated with elevated \( \text{HvFT1} \) expression levels. In contrast, \( \text{HoVRN1} \) accelerates inflorescence initiation independently of \( \text{PPD-H1} \) activity, and low-temperature induction of \( \text{HoVRN1} \) does not require \( \text{HoVRN2} \) or \( \text{PPD-H1} \).

**RESULTS**

**Genetic Interactions between \( \text{HoVRN2} \) and \( \text{PPD-H1} \)**

Influence Flowering Time in Barley

We examined flowering time (head emergence) in a Sloop × Halcyon doubled haploid population (Read et al., 2003). This population segregates for alleles of \( \text{HoVRN1} \) and \( \text{HoVRN2} \) (Read et al., 2003, Trevaskis et al., 2006; Table I). Lines that inherit both \( \text{HoVRN1} \) and \( \text{HoVRN2} \) alleles from the parent Halcyon are predicted to require vernalization and be late flowering in the absence of prolonged exposure to cold. The two loci are not linked, so the doubled haploid population (homozygous at all loci; Jain et al., 1996) is predicted to have a 3:1 ratio of early:late flowering in the absence of vernalization. When grown under long days in field experiments (Boyd et al., 2003) 56 of the 131 lines examined were late flowering (Supplemental Fig. S1). This result deviates significantly from the expected ratio of 3:1 (early:late; \( \chi^2, 21.6 \); see Supplemental Data S1), due to the larger-than-expected number of late-flowering lines. All the lines with the \( \text{HoVRN1-1} \) allele, which is expressed at high basal levels, flowered early, suggesting that expression of \( \text{HoVRN1} \) can activate flowering regardless of genotypes at other loci segregating in this population (Supplemental Fig. S1). In contrast, 22 of the 50 lines deleted for \( \text{HoVRN2} \) (\( \text{HoVRN1} \Delta \text{HoVRN2} \)), which were expected to be early flowering, flowered late (Supplemental Fig. S1). This suggests that alleles of another unlinked locus are segregating in the Sloop × Halcyon population and affect flowering time in lines that lack \( \text{HoVRN2} \).

A third major quantitative trait locus affecting flowering time in long days mapped to a region containing \( \text{PPD-H1} \) (chromosome 2H, unlinked to \( \text{HoVRN1} \) on chromosome 5H or \( \text{HoVRN2} \) on chromosome 4H) in the

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**Table I. Genotypes of flowering time genes for Halcyon and Sloop**

<table>
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<th>Description</th>
<th>Halcyon</th>
<th>Snoop</th>
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<tr>
<td>( \text{HvVRN1} ) genotype</td>
<td>Full-length ( \text{HvVRN1} ) gene, which is expressed at low basal levels.</td>
<td>( \text{HvVRN1-1} )</td>
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Sloop × Halycon population (Read et al., 2003). We used a molecular marker (see “Materials and Methods”) to genotype the Sloop × Halycon population for PPD-H1. This showed that Halcyon carries an inactive allele of PPD-H1 (ppd-H1) previously identified in daylength insensitive varieties (Turner et al., 2005) whereas Sloop carries an active allele (Table I). In lines deleted for HvVRN2 there was a complete association between PPD-H1 genotype and flowering time (Supplemental Fig. S1); lines with an active allele of PPD-H1 flowered early, whereas lines with the inactive ppd-H1 allele were late flowering. This explains the deviation from the expected ratio of 3:1 (early:late) in the Sloop × Halycon population.

Loss of HvVRN2 Causes Rapid Inflorescence Initiation and Increased HvFT1 Expression in Plants with the Active PPD-H1 Genotype

The interaction between HvVRN2 and PPD-H1 was examined further in controlled conditions. Ten lines from the Sloop × Halycon population, in which the HvVRN2 locus is deleted (HvVRN1;DHvVRN2), were grown in long days until flowering occurred (head

![Figure 1. Loss of HvVRN2 causes rapid inflorescence initiation and increased HvFT1 expression in plants with active PPD-H1 genotype. A, Heading date (average days to head emergence) of doubled haploid lines deleted for HvVRN2 (HvVRN1;ΔHvVRN2) and Halcyon grown in long-day glasshouse conditions. A, Active PPD-H1 allele. I, inactive ppd-H1 allele. Error bars represent s.e. B, Average length of the shoot apex in 14-d-old plants from the same lines. C, The shoot apices of representative lines carrying PPD-H1 or ppd-H1 alleles compared to Halcyon at different time points (days). DR, Double ridge, the first visible marker of inflorescence initiation. D, Expression levels of HvFT1 and HvVRN1 assayed by RT-PCR (40× cycles) in 7- and 14-d-old plants. Expression of ACTIN is shown as a loading comparison (25× cycles). E, Relative expression levels of HvFT1 and HvVRN1 in plants deleted for HvVRN2 but with different PPD-H1 genotypes. RNA from was pooled from four PPD-H1 lines or four ppd-H1 lines (same lines as in D) and expression levels were assayed by qRT-PCR and normalized to ACTIN. Asterisks indicate P value of Student’s t tests: **, P < 0.01. ***, P < 0.001. Error bars represent s.e of triplicate reactions. [See online article for color version of this figure.]
emergence). Two flowering-time classes were observed, and these correlated with the PPD-H1 genotype (Fig. 1A). Plants with the active PPD-H1 allele flowered early, whereas those with the inactive ppd-H1 allele flowered 4 weeks later. The early flowering phenotype of plants with the active PPD-H1 allele was caused by rapid shoot apex development, as indicated by apex length (Fig. 1B) and inflorescence initiation occurred within 13 d in all plants examined (Fig. 1C). In contrast, plants carrying the inactive ppd-H1 allele remained vegetative at the same time points (Fig. 1C). These results show that deletion of HvVRN2 promotes rapid inflorescence initiation only when combined with an active allele of PPD-H1.

Expression of HvFT1 was not detected in plants that carry HvVRN2 (HvVRN1;HvVRN2; see Fig. 4, D and E), consistent with previous reports that HvFT1 is expressed at low levels in vernalization requiring varieties (Yan et al., 2006). HvFT1 expression was also very low in plants deleted for HvVRN2 and carrying the inactive ppd-H1 allele (Fig. 1, D and E). In contrast, HvFT1 expression was detected in 7-d-old plants in lines carrying the active PPD-H1 allele (Fig. 1, D and E). This suggests that HvVRN2 normally down-regulates HvFT1 and counteracts activation of HvFT1 expression by PPD-H1. PPD-H1 expression levels were not correlated with HvVRN2 genotype (Supplemental Fig. S2). This suggests that HvVRN2 does not counteract activation of HvFT1 by PPD-H1 simply by repressing PPD-H1 expression. Expression of HvFT1 preceded any visible change in the morphology of the shoot apex (Fig. 1, C–E). HvVRN1 was not expressed at high levels in these plants at this early stage of development (7 d), but was expressed at high levels once the shoot apex progressed to inflorescence initiation (14 d; Fig. 1, C–E). These data suggest that increased expression of HvFT1 accelerates inflorescence initiation in lines with the HvVRN2 deletion allele and the active PPD-H1 allele, whereas HvVRN1 might only be induced as a consequence of increased HvFT1 expression or rapid floral development.

Overexpression of HvVRN2 Down-Regulates HvFT1 and Delays Flowering

The hypothesis that HvVRN2 down-regulates HvFT1 was tested by overexpressing HvVRN2 in transgenic overexpression construct (Ubi:VRN2) and sibling null controls (Null) in the T2 generation. Asterisks indicate P-values of Student’s t tests: ***, P < 0.001. B, Development of the shoot apex in transgenic plants homozygous for the HvVRN2 overexpression construct (Ubi:VRN2) and sibling null controls (Null), in the T2 generation grown in long-day glasshouse conditions. Apices were sampled at various time points (days). DR, Double ridge, the first visible marker of inflorescence initiation. C, Relative expression levels of HvVRN2, HvVRN1, and HvFT1 assayed by qRT-PCR and normalized to ACTIN in RNA from a transgenic plant homozygous for the HvVRN2 overexpression construct (Ubi:VRN2) and a sibling null control (Null), in the T2 generation grown in long-day glasshouse conditions. Error bars represent σ of triplicate reactions. ND, No expression was detected; NS, no significant difference between lines according to Student’s t tests. [See online article for color version of this figure.]
barley plants. A genomic copy of the HvVRN2 gene was expressed under the control of the constitutive maize (Zea mays) UBIQUITIN promoter in the barley cultivar Golden Promise. Golden Promise is deleted for HvVRN2 and carries the HvVRN1-1 allele. This allele has a deletion in the first intron of the HvVRN1 sequence and is expressed at high basal levels (see Table I), but the putative MADS-box binding site in the promoter is intact and expression of the HvVRN1-1 allele is induced by low temperatures (Trevaskis et al., 2007a). A single transgenic line was obtained, and in the descendent of this line a late-flowering phenotype segregated with the transgene. In the T2 generation, plants homozygous for the overexpression construct flowered 4 weeks later than sibling null controls (Fig. 2A). Inflorescence initiation was delayed by less than a week (Fig. 2B), so the delay in flowering observed was due mainly to slower development following inflorescence initiation. In 14-d-old seedlings, where the stage of apex development is similar in overexpression lines and in sibling null controls (Fig. 2B), HvFT1 expression levels in both the T1 and T2 generations were significantly lower in plants overexpressing HvVRN2 than in control lines (Fig. 2C). HvVRN1 expression levels did not differ between plants overexpressing HvVRN2 and sibling null control lines.

Figure 3. Expression of HvVRN1 promotes rapid inflorescence initiation independently of PPD-H1 genotype. A, Heading date (average days to head emergence) of doubled haploid lines with an allele of HvVRN1 that is expressed to high levels without vernalization (HvVRN1-1; HvVRN2) grown in long-day glasshouse conditions. A, Active PPD-H1 allele. I, Inactive ppd-H1 allele. Error bars represent SE. B, Average length of the shoot apex in 14-d-old plants from the same lines. C, The shoot apices of representative lines carrying PPD-H1 or ppd-H1 alleles at different time points (days). DR, Double ridge, the first visible marker of inflorescence initiation. D, Expression levels of HvFT1, HvVRN1, and HvVRN2 assayed by RT-PCR in 14-d-old plants (40 cycles). Expression of ACTIN is shown as a loading comparison (25 cycles). E, Relative expression levels of HvFT1 and HvVRN1 in plants with the HvVRN1 allele but with different PPD-H1 genotypes and relative expression levels of HvVRN2 in nonvernalized plants with the HvVRN1 allele and the HvVRN1-1 allele. RNA from was pooled from five PPD-H1 lines or five ppd-H1 lines (same lines as in D), then expression levels were assayed by qRT-PCR and normalized to ACTIN. Asterisks indicate P value of Student’s t tests: **, P < 0.01. NS, Not significant. Error bars represent si of triplicate reactions. [See online article for color version of this figure.]
Figure 4. *PPD-H1* genotype influences flowering time and *HvFT1* expression levels in vernalized plants. A, Heading date (average days to head emergence) in long-day glasshouse conditions for nonvernalized (NV) or vernalized (+V) plants from 10 vernalization-requiring doubled haploid lines (*HvVRN1; HvVRN2*). A, Active *PPD-H1* allele. I, Inactive *ppd-H1* allele. Error bars represent SE. B, Average length of the shoot apex in vernalized plants from the same lines. Plants were grown in long-day glasshouse conditions postvernalization, and were sampled after 1 week. C, The shoot apices of vernalized plants from representative lines. D, Expression levels of *HvFT1*, *HvVRN1*, and *HvVRN2* (40× cycles) assayed by RT-PCR in nonvernalized plants after 2 weeks in long-day glasshouse conditions and vernalized plants after 1 week in long-day glasshouse conditions. Expression of *ACTIN* is shown as a loading comparison (25× cycles). E, Relative expression levels of *HvFT1* and *HvVRN1* in nonvernalized and vernalized plants (*HvVRN1; HvVRN2*). RNA from was pooled from seven *PPD-H1* lines or three *ppd-H1* lines (same lines as in D), then expression levels were assayed by qRT-PCR and normalized to *ACTIN*. Asterisks indicate P value of Student’s t tests: ***, P < 0.001. NS, Not significant. Error bars represent SE of triplicate reactions. [See online article for color version of this figure.]
**HvVRN1 Promotes Inflorescence Initiation and Is Regulated by Low Temperatures Independently of HvVRN2 and PPD-H1**

Ten lines from the Sloop × Halcyon population carrying an allele of HvVRN1 that is expressed at high basal levels (HvVRN1-1; HvVRN2) were grown in long days. These lines showed inflorescence initiation within 16 d and took an average of 48 d to flower (Fig. 3, A and C). Flowering occurred approximately 14 d earlier in lines carrying the active PPD-H1 allele than in lines with the inactive ppd-H1 allele (Fig. 3A). This was due mainly to accelerated development after inflorescence initiation, as the timing of inflorescence initiation differed by less than 3 d between lines with the different PPD-H1 genotypes (Fig. 3, B and C). In 14-d-old plants, HvFT1 was expressed at higher levels in lines carrying the active PPD-H1 allele than in lines with the inactive ppd-H1 allele (Fig. 3, D and E), and this may account for the differences in apex development and flowering time. Expression of HvVRN2 was repressed in lines expressing HvVRN1 (Fig. 3, D and E). These data show that expression of HvVRN1 is sufficient to repress HvVRN2 and promote inflorescence initiation, and that this occurs regardless of PPD-H1 genotype.

Ten late-flowering lines from the Sloop × Halcyon population (HvVRN1; HvVRN2) were vernalized and transferred to long days. Flowering occurred approximately 8 weeks earlier than in nonvernalized plants (Fig. 4A). Two flowering times were observed among the vernalized plants and these correlated with PPD-H1 genotype (Fig. 4A). Apex development was more rapid and flowering occurred on average 16 d earlier in plants carrying the active PPD-H1 allele than in plants carrying the inactive ppd-H1 allele (Fig. 4, A–C). In all 10 lines, HvVRN1 and HvFT1 were induced by vernalization and HvVRN2 was repressed, regardless of PPD-H1 genotype (Fig. 4, D and E). HvFT1 expression levels in vernalized plants did correlate with the PPD-H1 genotype (Fig. 4, D and E) and this may account for the differences in apex development and flowering time.

**Figure 5.** HvVRN1 is induced by low temperatures in the absence of HvVRN2. A, Heading date (average days to head emergence) in long-day glasshouse conditions for nonvernalized (NV) and vernalized (+V) plants from lines deleted for HvVRN2 and carrying an inactive ppd-H1 allele (HvVRN1; ΔHvVRN2; ppd-H1). B, Average length of the shoot apex in the same lines, in 14-d-old nonvernalized plants, compared to vernalized plants that had grown in long-day glasshouse conditions for 1 week postvernalization to reach a similar stage of development (leaf number). C, Apices from representative nonvernalized and vernalized plants at the same time points. DR, Double ridge, the first visible marker of inflorescence initiation. D, Expression levels of HvFT1 and HvVRN1 assayed by RT-PCR in vernalized plants (HvVRN1; ΔHvVRN2; ppd-H1); expression levels of HvFT1 and HvVRN1 in nonvernalized plants at the same developmental stage [Zadoks scale 12] are presented in Figure 1; 40× cycles). Expression of ACTIN is shown as a loading comparison (25× cycles). [See online article for color version of this figure.]
Lines that lack *HvVRN2* and are daylength insensitive (*HvVRN1*; *DHvVRN2*; *ppd-H1*), which are late flowering without vernalization were also vernalized. Vernalization accelerated apex development in lines with this genotype, and head emergence occurred approximately 2 weeks earlier than in nonvernalized plants (Fig. 5, A–C). The flowering time of vernalized plants deleted for *HvVRN2* (average of 60 d) was similar to that of vernalized plants carrying *HvVRN2* (average of 57 d), so *HvVRN2* genotype has little influence on flowering time in vernalized plants.

*HvVRN1* expression was induced by vernalization in all these lines (Fig. 5D). This shows that *HvVRN1* is induced by prolonged exposure to low temperatures in the absence of *HvVRN2*, and this may account for the acceleration of flowering in these lines. There was variation in *HvFT1* expression levels in vernalized plants deleted for *HvVRN2* (Fig. 5D), suggesting that segregation of another locus might influence *HvFT1* expression when plants with this genotype are vernalized.

**HvFT1** Does Not Influence the Vernalization Requirement in the Sloop × Halcyon Population and Expression of *HvFT1* Does Not Increase during Low-Temperature Treatment in Short Days

Single nucleotide changes in the first intron of *HvFT1* may provide the molecular basis for dominant alleles of VRN3 that reduce the vernalization requirement (Yan et al., 2006). The sequence of the *HvFT1* first intron from Sloop is identical to that isolated from barley varieties that have dominant alleles of VRN3 that reduce the vernalization requirement, whereas the *HvFT1* first intron sequence from Halcyon is identical to that found in barley varieties that require vernalization (Supplemental Fig. S3). In the Sloop × Halcyon population there was no association between these different *HvFT1* sequences and vernalization requirement (Fig. 6), and *HvFT1* was not expressed in nonvernalized plants, regardless of which *HvFT1* sequence was present (Fig. 4, D and E). Consistent with these observations, no quantitative trait loci for flowering time have been associated with the VRN3 region in the Sloop × Halcyon population (Read et al., 2003).

It has been suggested that expression of *HvFT1* is induced by low temperatures, and that this plays a role in the vernalization response in cereals (Yan et al., 2006). Expression of *HvFT1* was examined in a vernalization-requiring, daylength-sensitive barley variety (Sonja *HvVRN1;HvVRN2;PPD-H1*). In nonvernalized plants grown in short days, expression levels of *HvFT1* were below the limit of detection by quantitative real-time PCR (qRT-PCR). There was no detectable increase in *HvFT1* expression when these plants were exposed to long days (Fig. 7, A and B).

![Figure 6](image-url)  
*Figure 6.* Single nucleotide polymorphisms in the first intron of *HvFT1* do not influence the vernalization requirement in the Sloop × Halcyon population. *HvFT1* genotype, scored by CAPS marker, in 10 vernalization-requiring lines (*HvVRN1;HvVRN2*; see “Materials and Methods”). *H*, Halcyon allele; *S*, Sloop allele. Flowering time (days to head emergence) for nonvernalized (NV) and vernalized (+V) plants in long days in glasshouse conditions.

![Figure 7](image-url)  
*Figure 7.* *HvFT1* is not induced by low temperatures alone. A, Relative expression levels of *HvFT1* assayed by qRT-PCR and normalized to *ACTIN* in RNA from nonvernalized plants (Sonja, minus roots) that were grown for 2 weeks in short days then the following: maintained in short days for 2 weeks (SD), shifted to long days for 2 weeks (LD), vernalized for 9 weeks in short days and harvested directly from vernalization chamber (SDV), vernalized in short days for 9 weeks then returned to glasshouse temperatures in long days for 2 weeks (V + LD). Error bars represent sd of triplicate reactions. B, Expression levels of *HvVRN1* and *HvFT1* assayed by RT-PCR in the same samples (40× cycles). Expression of *HvVRN1* is shown as a positive control for the vernalization treatment. Expression of *ACTIN* is shown as a loading comparison (25× cycles).
Expression of HoFT1 also remained low in plants that were maintained in long days without vernalization (Supplemental Fig. S4). To test whether HoFT1 is induced by low-temperature, HoFT1 expression levels were monitored at weekly intervals in plants grown at vernalizing temperatures in short days. HoFT1 expression remained below the limit of detection throughout the 9-week vernalization treatment. Following vernalization, expression of HoFT1 remained low in plants that were maintained in short days, but was induced when vernalized plants were exposed to long days (Fig. 7, A and B). These data show that HoFT1 is not induced by cold per se, but by long days when the vernalization requirement has been satisfied.

**DISCUSSION**

By analyzing flowering time in a doubled haploid barley population that segregates for genes controlling vernalization requirement and daylength sensitivity, we found that a deletion of HoVRN2 causes early flowering only when an active allele of PPD-H1 is present. This genetic interaction between HoVRN2 and PPD-H1 has not been described previously and has implications for our understanding of how different genetic pathways regulate flowering time in cereals.

**Deletion of HoVRN2 Allows Expression of HoFT1 to Induce Flowering without Vernalization**

Vernalization accelerates flowering by promoting inflorescence initiation, the transition to reproductive development at the shoot apex (Flood and Halloran, 1984). Our data show that deletion of HoVRN2 can bypass the vernalization requirement and promote rapid inflorescence initiation, but only when combined with the active PPD-H1 genotype. PPD-H1 mediates long-day induction of HoFT1 (Turner et al., 2005), and lines that combine deletion of HoVRN2 with an active allele of PPD-H1 have elevated HoFT1 expression levels. This suggests that HoVRN2 down-regulates expression of HoFT1 and counteracts PPD-H1. Thus, HoVRN2 regulates flowering by repressing the activity of the long-day response pathway. This proposed role is consistent with HoVRN2 being expressed only in long days (Dubcovsky et al., 2006; Trevaskis et al., 2006), and explains why lines that lack HoVRN2 flower early only in long days (Karsai et al., 2005). HoVRN2 may interact directly with components of the long-day response pathway to regulate HoFT1 expression. HoVRN2 has a CCT domain, a type of domain known to interact with CCAAT binding factors (Ben-Naim et al., 2006). In Arabidopsis a CCAAT binding factor, HAP3b, has been shown to up-regulate FT and promote flowering in long days (Cai et al., 2007). HoVRN2 might interact negatively with a HAP3b-like factor in cereals to repress HoFT1 expression. If PPD-H1, which also contains a CCT domain, interacts with the same HAP3b-like factor to enhance HoFT1 expression this could explain the antagonistic relationship between HoVRN2 and PPD-H1.

Both FT1 and VRN1 are expressed at elevated levels in early flowering wheats that lack VRN2 (Yan et al., 2003, 2006). The strong influence of PPD-H1 (a known regulator of HoFT1) on flowering time in barley lines that lack HoVRN2 suggests that elevated expression of HoFT1 is the primary cause of early flowering in lines that lack HoVRN2. Expression data support this conclusion. HoFT1 is expressed at high levels during early stages of vegetative development in barley plants that lack HoVRN2 and have the active PPD-H1 allele, whereas expression of HoVRN1 increases around the time of inflorescence initiation. Deletion of HoVRN2 may indirectly cause increased HoVRN1 expression by promoting floral development because HoVRN1 expression is induced during inflorescence development (Trevaskis et al., 2007a) and is required for inflorescence initiation (Shitzukawa et al., 2007). Consistent with this idea, HoVRN1 expression levels are not elevated in lines that combine the inactive ppd-H1 allele with deletion of HoVRN2, where reproductive development of the shoot apex is delayed. HoVRN1 might also be induced in leaves as a consequence of HoFT1 induction. In Arabidopsis, activation of FT leads to induction of FRUITFUL (the Arabidopsis ortholog of VRN1) in leaves (Corbesier et al., 2007). In summary, HoVRN2 is likely to delay flowering by down-regulating expression of HoFT1.

**Figure 8.** Interactions between genetic pathways controlling seasonal induction of flowering in temperate cereals. VRN2 represses FT1 to counteract PPD1 dependent long-day induction of FT1 prior to winter. Prolonged exposure to low temperatures up-regulates VRN1, which promotes inflorescence meristem identity at the shoot apex, accelerating inflorescence initiation independently of the long-day response pathway. VRN1 also represses VRN2 in the leaves to allow the long-day induction of FT1. FT1 can further accelerate inflorescence initiation and also subsequent stages of inflorescence development. According to this model, VRN1 acts in a low-temperature response pathway, FT1 acts in a daylength response pathway, and VRN2 integrates the low-temperature and daylength responses. In varieties where VRN2 is deleted, or where dominant VRN3 alleles are present, increased FT1 expression accelerates inflorescence initiation. This induces expression of VRN1 (dotted line).
HoVRN1 Promotes Inflorescence Initiation and Represses HoVRN2 to Allow Expression of HoFT1

The HoVRN1-1 allele, which is expressed at high basal levels, accelerates inflorescence initiation and promotes flowering. Increased HoVRN1 expression probably promotes inflorescence initiation by promoting inflorescence meristem identity at the shoot apex. Expression of HoVRN2 was repressed in lines that carry HoVRN1-1 (as reported previously; Trevaskis et al., 2006). Consistent with the function proposed for HoVRN2, expression of HoFT1 was elevated in these lines and was highest in lines carrying the active allele of PPD-H1. Taken together, the molecular phenotypes of plants carrying the HoVRN1-1 allele are very similar to those of vernalized plants. This shows that, unlike deletion of HoVRN2, which seems to activate flowering in long days through a daylength response pathway, the HoVRN1-1 allele can substitute for prolonged exposure to cold.

Previous interpretations of genetic interactions between VRN1 and VRN2 have led to the suggestion that VRN1 genotype does not influence flowering time in lines that lack VRN2 (Yan et al., 2006). Our data show this is not the case; the HoVRN1-1 allele is associated with elevated HoVRN1 expression, accelerated inflorescence initiation, and early flowering in the absence of HoVRN2 (see ΔHoVRN2/ppd-H1/HoVRN1 versus ΔHoVRN2/ppd-H1/HoVRN1-1; Supplemental Figs. S1 and S5).

The Molecular Basis of the Vernalization Response

When studying the vernalization response it is important to differentiate between the response to low temperatures, which occurs during winter when days are short, and the response to long days, which occurs after winter. When plants are vernalized in short days (Fig. 7), HoVRN1 is induced by cold without changes in expression levels of HoFT1 or HoVRN2. Furthermore, low-temperature induction of HoVRN1 can occur in the absence of active versions of HoVRN2 or PPD-H1 (Figs. 4 and 5). This shows that HoVRN1 is regulated by a low-temperature response pathway that operates independently of the daylength response. We suggest that vernalization and daylength response pathways act sequentially to promote flowering. Before vernalization, both the daylength and vernalization pathways are inactive; expression of HoVRN1 is low, and HoVRN2 blocks long-day induction of HoFT1. During vernalization HoVRN1 expression is induced. Following vernalization, HoVRN1 is expressed and HoVRN2 is repressed, allowing long-day induction of HoFT1. According to this model, HoVRN1 acts in the vernalization response pathway, HoFT1 acts in the daylength response pathway, and HoVRN2 is an integrator of the vernalization and daylength responses (Trevaskis et al., 2007b; Fig. 8).

Our data show that expression of HoVRN1 can promote inflorescence initiation, whereas expression of HoFT1 can accelerate both inflorescence initiation and subsequent stages of inflorescence development. These observations are consistent with studies showing that low temperatures can accelerate flowering until plants reach the double ridge stage, whereas long days can accelerate flowering until the terminal spikelet differentiates (Flood and Halloran, 1984; Roberts et al., 1988). Low temperatures and long days are likely to promote flowering by accelerating different stages of floral development. Induction of HoVRN1 during winter will promote inflorescence initiation. Subsequently, as daylength increases, expression of HoFT1 will accelerate later stages of inflorescence development.

Another model for the vernalization response in cereals suggests that VRN2 is a repressor of VRN1 and FT1 (VRN3), and that VRN2 is down-regulated by cold or daylength to elicit the response to low temperature during winter (Yan et al., 2004, 2006). Our model differs in that it proposes that VRN1 is induced by low temperature independently of VRN2 and FT1, which regulate the daylength response. Both models offer similar mechanisms to account for natural variation in the vernalization requirement; alleles of VRN1 and VRN3 with high basal expression levels, or deletion of VRN2, promote flowering and remove the requirement for vernalization. The molecular basis of dominant alleles of VRN3 in barley is unclear. The Sloop × Halcyon population segregates for two HoFT1 sequences suggested to correspond to the different alleles of VRN3 (Yan et al., 2006), but HoFT1 genotype did not affect vernalization requirement in this population. Sequence changes outside the proximal promoter and transcribed region that influence HoFT1 expression may provide the molecular basis for the dominant VRN3 allele in the mapping population used by Yan et al. (2006). A third model for the vernalization response of cereals is that low temperatures repress VRT2, a MADS-box gene suggested to repress VRN1, and this allows expression of VRN1 to increase (Kane et al., 2005, 2007). This model seems unlikely because in barley HoVRT2 is induced by low temperatures (Trevaskis et al., 2007a).

Understanding how genes that regulate the vernalization and daylength responses interact to control flowering is important for cereal breeding. The strong genetic interaction between HoVRN2 and PPD-H1 has implications for temperate cereal breeding strategies; PPD-H1 genotype is likely to be important when HoVRN2 is used to breed for a reduced vernalization requirement and early flowering.

MATERIALS AND METHODS

Plant Growth Conditions

The field flowering time experiments have been described previously (Boyd et al., 2003). Glasshouse experiments were planted in January, 2007. For glasshouse experiments, plants were grown in pots of soil in sunlight.
houses under long days (16-h light/8-h dark) with supplementary lighting when natural light levels dropped below 200 μE. Glasshouses had an average temperature of 19°C. Plants were harvested at the middle of the light period for short day treatments, or 12 h after the beginning of the light period (mid-afternoon) for long day treatments. For vernalization treatment, seeds were imbibed in foil-covered pots at 4°C for 10 weeks. Pots were then moved to standard glasshouse conditions for 1 week. At this stage, leaf number was similar to that of 14-d-old seedlings grown in standard glasshouse conditions. Experiments were terminated after 120 d of growth in glasshouse conditions.

**Apex Length and Flowering Time Measurements**

Apices were isolated under a binocular dissecting microscope and then digitally photographed on a Leica M8 digital camera. Apex lengths were measured from digital images using analySIS LS Professional (Olympus Soft Imaging Solutions). Apex lengths were averaged from three to six plants. Headling date was measured as the day when the head first emerged from the sheath, and averaged for five plants.

**DNA and RNA Extraction**

DNA for PCR genotyping was extracted from individual dry seeds in a 96-well format. Seeds were ground in an extraction buffer, (2% cetyl trimethylammonium bromide, 2% polyvinylpyrrolidone, 0.02% EDTA, 1.4% NaCl, 100 mM Tris-Cl, pH 8.0) using a ball bearing in a mix mill. Extracts were incubated at 65°C for 30 min then cleared by centrifugation. DNA was precipitated with 1 vol of isopropanol and 0.1 vol of 6 M ammonium acetate, then centrifuged. Pellets were washed in 70% ethanol and resuspended in 150 μL of Tris-EDTA buffer (10 mM Tris-Cl, pH 8, 1 mM EDTA). Total RNA was extracted from whole seedlings, excluding root tissue, using the method of Chang et al. (1993).

**Genotyping**

Plants were genotyped for HvVRN1 and HvVRN2 (HvZCCTb) as described previously (Trevaskis et al., 2008). Plants were genotyped for PPD-HI with a cleaved amplified polymorphic sequence (CAPS) marker using primers 5′-AACACCAATAAAGCGGCAAC-3′ and 5′-GTCGCCGGGATGTTACCT-3′ and BstUI. Plants were genotyped for HvFTI with a CAPS marker amplified using primers 5′-ACTTGGAACATCTGGTTCAC-3′ and 5′-CACTTTTCTGTCCTGTTTAGT-3′, then digested with the BseI enzyme.

**Reverse Transcription-PCR and qRT-PCR**

To remove genomic DNA contamination, RNA was treated with RQ1 RNase-free DNase (Promega) according to the manufacturer’s instructions. An oligo(T) primer (T18[G/C/A]) was used to prime first-strand complementary DNA synthesis from 5 μg of total RNA using the SuperScript III reverse transcriptase enzyme (Invitrogen) according to the manufacturer’s instructions. A single reverse transcription (RT) reaction was performed for each RNA sample. RT-PCR was performed using Taq DNA polymerase (Fischer Biotech). The primers used for HvFTI, HvVRN1, HvVRN2 (HvZCCTb), and ACTIN have been described previously (Trevaskis et al., 2006). The primers used for PPD-HI were 5′-CAACCAATAAAGCGGCAAC-3′ and 5′-GTCGCCGGGATGTTACCT-3′. Each primer pair amplifies DNA-specific DNA products. Cycling conditions were 40 cycles (except for ACTIN, 25 cycles) 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C. Fragments were visualized by agarose gel electrophoresis.

qRT-PCR was performed on a Rotor-Gene 3000 real-time cycler (Corbett Research) with the same primer pair set. qRT-PCR was performed using Platinum Taq DNA polymerase (Invitrogen). Cycling conditions were 2 min at 95°C, 50 cycles of 10 s at 95°C, 15 s at 60°C, and 20 s at 72°C. This was followed by a melting-curve program (72°C–95°C with a 5°C hold at each temperature). Fluorescence data were acquired at the 72°C step and during the melting curve program. Expression levels of genes of interest relative to ACTIN were calculated using the comparative quantitative analysis method (Rotogène-5; Corbett Research), which takes into account the amplification efficiency of each primer set. Quantification for each primer set and cDNA template combination was performed in triplicate, and included a no-template control, to ensure results were not influenced by primer-dimer formation or DNA contamination. Data presented are the average and SE from triplicate reactions on the same PCR run.

**Plant Transformation**

An HvVRN2 overexpression construct was made using the GATEWAY cloning system. An HvZCCTb gene fragment was inserted into an entry vector and then recombined into the pUBI-GATEWAY vector, a vector constructed with a GATEWAY destination site downstream of the maize (Zea mays) UBQ10/LEUM promoter (Christensen et al., 1992) in the pWBVEC8 binary vector backbone (Wang et al., 1998). barley (Hordeum vulgare) plants were transformed using Agrobacterium transformation of excised embryos (Tingay et al., 1999; Mathews et al., 2001). T1 and T2 plants were screened for segregation of the transgene using primers that amplify from the hygromycin selectable marker gene. Expression analysis was carried out on plants hemizygous or homozygous for the transgene and sibling null control lines that did not inherit the transgene.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** HvVRN1, HvVRN2, and PPD-HI genotype versus flowering time, from a field planting of the Sloop × Halcyon doubled haploid population.

**Supplemental Figure S2.** Relative expression levels of PPD-HI in lines carrying HvVRN2 versus lines deleted for HvVRN2.

**Supplemental Figure S3.** Nucleotide sequence alignment showing allelic variation in HvFTI in Halcyon and Sloop.

**Supplemental Figure S4.** A time course of HvFTI expression in non-vernalyzed plants growing in long days.

**Supplemental Figure S5.** The shoot apices of representative lines deleted for HvVRN2 and carrying the inactive ppd-H1 allele, but with different HvVRN1 genotypes.

**Supplemental Data S1.** Genetics of flowering time in the Sloop × Halcyon doubled haploid population.

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**LITERATURE CITED**


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