HvVRN2 Responds to Daylength, whereas HvVRN1 Is Regulated by Vernalization and Developmental Status\(^1\)

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Two genetic loci control the vernalization response in winter cereals; VRN1, which encodes an AP1-like MADS-box transcription factor, and VRN2, which has been mapped to a chromosome region containing ZCCT zinc finger transcription factor genes. We examined whether daylength regulates expression of HvVRN1 and HvVRN2. In a vernalization-responsive winter barley (Hordeum vulgare), expression of HvVRN1 is regulated by vernalization and by development, but not by daylength. Daylength affected HvVRN1 expression in only one of six vernalization-insensitive spring barleys examined and so cannot be a general feature of regulation of this gene. In contrast, daylength is the major determinant of expression levels of two ZCCT genes found at the barley VRN2 locus, HvZCCTa and HvZCCTb. In winter barley, high levels of HvZCCTa and HvZCCTb expression were detected only when plants were grown in long days. During vernalization in long-day conditions, HvVRN1 is induced and expression of HvZCCTb is repressed. During vernalization under short days, induction of HvVRN1 occurs without changes in HvZCCTa and HvZCCTb expression. Analysis of HvZCCTa and HvZCCTb expression levels in a doubled haploid population segregating for different vernalization and daylength requirements showed that HvVRN1 genotype determines HvZCCTa and HvZCCTb expression levels. We conclude that the vernalization response is mediated through HvVRN1, whereas HvZCCTa and HvZCCTb respond to daylength cues to repress flowering under long days in nonvernalized plants.

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flowering in plants that have not been vernalized and explains the dominant gene action of VRN1 spring alleles. In hexaploid wheat (Triticum aestivum), dominant spring alleles of VRN1 have been identified in all three genomes (Pugsley, 1971). Different spring homeo-alleles of VRN1 cause different levels of VRN1 expression in nonvernalized plants and have different effects on flowering time (Murai et al., 2003; Trevaskis et al., 2003). Mutations in the promoter or first intron of VRN1 have been identified in all three genomes (Pugsley, 1971). Different spring homeo-alleles of VRN1 cause different levels of VRN1 expression in nonvernalized plants and have different effects on flowering time (Murai et al., 2003; Trevaskis et al., 2003). Mutations in the promoter or first intron of the VRN1 gene sequence are associated with spring alleles of VRN1. These regions may mediate repression of VRN1 in nonvernalized plants (Yan et al., 2004a; Fu et al., 2005).

Expression of VRN1 can also be regulated by daylength. The barley VRN1 gene, HvVRN1 (see note on gene nomenclature in “Materials and Methods”), is strongly induced by long days in the daylength-responsive barley cultivar Dicktoo (Danyluk et al., 2003). Likewise, the VRN1 gene is up-regulated by long days in spring wheat (Murai et al., 2003) and the vernalization-insensitive rye grass Lolium temulentum (Gocal et al., 2001). It is possible that VRN1 expression integrates vernalization and daylength response pathways such that VRN1 expression is first derepressed by vernalization (or by mutations in spring VRN1 alleles), then further activated by long days to accelerate flowering in the spring. How VRN1 expression is activated in response to vernalization or daylength cues is unclear.

One potential regulator of VRN1 is the VRN2 gene (Yan et al., 2004b). Genetic data show that VRN2 acts as a repressor of flowering in plants that have not been vernalized (Takahashi and Yasuda, 1971; Dubcovsky et al., 1998; Karsai et al., 2005). Two genes (ZCCT1 and ZCCT2) have been identified at the wheat VRN2 locus. These encode proteins with a zinc finger domain and a CCT (CONSTANS, CONSTANS-like, and TOC) domain (Yan et al., 2004b). The ZCCT1 gene is expressed in the leaves and apices of plants that have not been vernalized, but is repressed during vernalization. Loss-of-function mutations in ZCCT1 are associated with the early flowering VRN2 spring habit phenotype (Yan et al., 2004b; Dubcovsky et al., 2005) and silencing of VRN2 (ZCCT1) in winter wheat (cv Jagger) reduces the time taken to flower, consistent with VRN2 acting as a repressor of flowering (Yan et al., 2004b). Plants with reduced VRN2 expression have increased levels of VRN1. A two-gene model for the vernalization response pathway in winter cereals has been suggested on the basis of these data. According to this model, VRN2 acts to delay flowering before vernalization by repressing VRN1 expression, but vernalization represses VRN2 (ZCCT1) and enables induction of VRN1 to promote flowering (Yan et al., 2004b). This model is consistent with genetic data that show epistatic interactions between VRN1 and VRN2 (Tranquilli and Dubcovsky, 1998).

The model for the molecular basis of the vernalization response in winter cereals outlined above does not address the potential for daylength regulation of VRN1 or VRN2. We have examined regulation of HvVRN1 and of ZCCT genes found at the HvVRN2 locus of barley (see note on gene nomenclature in “Materials and Methods”) by both vernalization and daylength. We show that HvVRN1 is regulated primarily by vernalization and developmental cues, whereas daylength is the major determinant of HvZCCTa and HvZCCTb gene expression, and on the basis of these data we suggest an alternative model for the molecular basis of the vernalization response in winter cereals.

RESULTS

HvVRN1 Is Regulated by Development and Vernalization, But Not by Daylength, in Most Barley Varieties

The effect of daylength on HvVRN1 expression was initially examined in vernalization and daylength-responsive winter barley (cv Sonja). Plants (nonvernalized) were grown under either long or short days and HvVRN1 expression levels in plants from each daylength condition were compared at a number of time points. HvVRN1 expression was initially low in both conditions, but increased gradually during development (Fig. 1A). HvVRN1 expression increased more rapidly in long days. Expression of HvVRN1 was detectable by RNA gel blots after 10 weeks in long-day-grown plants, but was only faintly detectable after 12 weeks in short-day-grown plants. This correlates with earlier flowering in long-day conditions (Fig. 1B). The effect on HvVRN1 expression of shifting plants from short days to long days was also examined. Plants were grown under short days for 21 d, then either kept in short days or shifted to long days. HvVRN1 expression was not detected by RNA gel-blot analysis in plants after 14 d in either treatment, but weak expression of HvVRN1 could be detected by real-time reverse transcription (RT)-PCR. The level of expression was similar in both daylength treatments (Fig. 1C).

HvVRN1 expression can be induced in this winter barley by vernalization (Trevaskis et al., 2003). The effects of different daylengths on the induction of HvVRN1 by vernalization were examined. Plants (21-d-old) were vernalized under either long or short daylengths for up to 9 weeks, and HvVRN1 expression levels were compared. Strong induction of HvVRN1 was evident after 7 weeks of vernalization in both daylength conditions (Fig. 1D). Real-time RT-PCR analysis of HvVRN1 expression levels showed that at the end of the vernalization treatment induction of HvVRN1 was slightly higher in plants vernalized under short days (Fig. 1E). Expression of HvVRN1 increased further when plants were returned to glasshouse temperatures regardless of daylength conditions (Fig. 1E).

Regulation of HvVRN1 expression by daylength was examined in seven spring barley varieties that express HvVRN1 without any requirement for vernalization. HvVRN1 expression levels were compared in plants that had been grown for 14 d in either long- or...
short-day conditions. In six varieties (Chame 11, Sikangense, Himalayense type 15, Golden Promise, Morex, and Olli) daylength had no effect on HvVRN1 expression. In one variety (cv Icheon Naked), HvVRN1 expression was higher in long-day-grown plants (Fig. 2). All varieties flowered earlier under long days than short days and Icheon Naked flowered approximately 2 weeks earlier in long days than other spring barleys with the same genotypes for HvVRN1 and HvVRN2.

Expression of HvZCCTa and HvZCCTb Is Daylength Responsive

Two homologs of the T. monococcum VRN2 gene (TmZCCT1) have been identified at the orthologous HvVRN2 locus of barley. These genes have been designated HvZCCTa and HvZCCTb (Yan et al., 2004b). A partial sequence has also been obtained for a third ZCCT-like gene, which may be located in the same chromosomal region (Dubcovsky et al., 2005). Typically, all three HvZCCT genes are deleted in spring barleys that carry the recessive spring habit genotype at the HvVRN2 locus (Hvvrn2 genotype; Dubcovsky et al., 2005). To determine whether these genes, HvZCCTa, HvZCCTb, and HvZCCTc, are regulated by daylength, the effect of short or long daylength regimes on gene expression was examined in winter barley (cv Sonja). An antisense riboprobe, predicted to hybridize to all three HvZCCT genes, detected expression of HvZCCT genes in long-day-grown plants (Fig. 3A). RT-PCR with gene-specific primers showed that...
both \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) are expressed in long-day-grown plants (Fig. 3B). In short-day-grown plants (21-d-old), expression of both \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) was low and could not be detected using RT-PCR with gene-specific primers (Fig. 3B). Expression of \( \text{HvZCCTc} \) could not be detected in either treatment.

The effects of changes in daylength on \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) expression were then examined using RT-PCR with gene-specific primers. Plants were grown for 21 d under short days, then shifted to long days and harvested at different time points. Exposure to a single long day induced expression of both \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) (Fig. 4A). Expression remained high in the plants shifted to long days at all subsequent time points analyzed (Fig. 4A). The increase in \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) transcript levels in plants shifted to long-day conditions showed no relationship with \( \text{HvVRN1} \) expression levels, which remained constant (Fig. 1C).

Because \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) have similar responses to daylength, changes in expression levels of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) were quantified using RT-PCR with highly efficient primers that amplify both genes. In short-day-grown plants, expression of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) was weak. Within the period of a single long-day treatment, expression of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) increased dramatically (Fig. 4B). These results are consistent with those obtained for each gene independently using gene-specific primers (Fig. 4A). A rapid decrease in expression of both \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) occurred when plants were shifted from long days to short days in both glasshouse and vernalizing conditions (Fig. 4C). After a week in short-day conditions, expression of both \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) was too low to be detected by RT-PCR with gene-specific primers regardless of temperature. This suggests that the effect of daylength on \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) expression overrides that of temperature.

A feature of many daylength-responsive genes is an expression pattern that follows a diurnal rhythm. The expression levels of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) were examined at 2-h intervals throughout a 24-h cycle in non-vernalized plants grown under long- or short-day conditions. Under long days, expression of these genes showed a strong diurnal rhythm (Fig. 5). Maximal expression levels were observed at the end of the light period and expression decreased with the onset of darkness. Expression levels remained low throughout the dark period, but increased when the plants were reexposed to light. Using gene-specific RT-PCR primers, expression of both \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) could be detected at the time points when high expression levels were detected by quantitative RT-PCR. In short days, expression of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) remained low throughout the 24-h cycle (Fig. 5).

Vernalization of Plants in Short-Day Conditions Induces \( \text{HvVRN1} \) and Promotes Flowering, whereas Expression of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) Remains Low

Vernalization of plants in short days promotes flowering (Fig. 1B). \( \text{HvVRN1} \) was induced by this treatment (Fig. 1, D and E), but the expression levels of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) remained low (Fig. 6). This suggests that repression of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) may not be associated with induction of \( \text{HvVRN1} \) by vernalization in short-day conditions. Vernalization did not block induction of \( \text{HvZCCTa} \) or \( \text{HvZCCTb} \) by long days. Expression of both genes increased when plants that had been vernalized under short days were moved to normal glasshouse temperatures with long days (Fig. 6).

Expression of \( \text{HvZCCTb} \) Is Regulated by Vernalization in Long Days in Winter Barley

Expression of \( \text{HvZCCTa} \) and \( \text{HvZCCTb} \) was examined during vernalization under long days using...
real-time RT-PCR. The total level of HvZCCTa and HvZCCTb expression was lower in vernalized plants than in control plants grown in glasshouse conditions for the same length of time (Fig. 7A). RT-PCR with gene-specific primers showed that the drop in expression detected by quantitative RT-PCR was due primarily to a decrease in HvZCCTb expression during the course of the vernalization treatment (Fig. 7B). Expression of both genes remained high in control plants maintained under normal long-day glasshouse temperatures for the same period of time (Fig. 7B). The decrease in expression of HvZCCTb during vernalization under long days is similar to that reported previously for the ZCCT1 and ZCCT2 genes of T. monococcum during vernalization under similar daylength conditions (Yan et al., 2004b).

**HvVRN1 Genotype Is a Major Determinant of HvZCCTa and HvZCCTb Expression in Nonvernalized Plants**

Regulatory interactions between HvZCCTa, HvZCCTb, and HvVRN1 were examined in nonvernalized plants from a doubled haploid population derived from a cross between winter barley (cv Halcyon) and spring barley (cv Sloop). This population segregates for vernalization requirements at both HvVRN1 and HvVRN2 (Read et al., 2003). As expected, no expression of either HvZCCTa or HvZCCTb was observed in lines carrying the deletion of the HvVRN2 locus. All lines with the HvVRN1 spring habit genotype also had low levels of HvZCCTb expression, below the limit of detection by RT-PCR (Fig. 8). Expression of HvZCCTa was also low. Expression of both the HvZCCTa and HvZCCTb genes was only observed in barleys with the winter HvVRN1 and winter HvVRN2 genotypic combination (Hvvrn1/HvVRN2). This suggests that the HvVRN1 genotype determines HvZCCTa and HvZCCTb expression levels in nonvernalized plants.

**DISCUSSION**

By examining the effects of different combinations of daylength and vernalization treatments on expression of HvVRN1 and HvVRN2 (HvZCCTa and HvZCCTb) in barley, we have found that these genes are, to a large extent, regulated by different environmental cues. Typically, vernalization had the greatest effect on HvVRN1 expression, whereas daylength had the greatest effect on expression of HvZCCTa and HvZCCTb. For both genes, however, the effect of each environmental cue is dependent on the genetic background of the varieties tested.

In a vernalization-responsive barley cultivar (cv Sonja), vernalization had the largest effect on HvVRN1,
Expression of *HvVRN1* was not affected by daylength in most spring varieties examined. Daylength activation of *HvVRN1* expression occurred in only one of the seven spring barleys examined (Fig. 2). The daylength response of *HvVRN1* expression seen in Icheon Naked and reported for Dicktoo (Danylik et al., 2003) may be due to specific combinations of genotypes at photoperiod and vernalization loci. Both Dicktoo and Icheon Naked are photoperiod responsive and both have the *HvVRN2* spring habit genotype (*Hvvrn2*). The combination of a strong photoperiod response and the absence of *HvVRN2* function may be a prerequisite for induction of *HvVRN1* by long days. Alternatively, the daylength induction of *HvVRN1* in these varieties may be a property of the *HvVRN1* alleles that they carry.

For the *HvZCCTa* and *HvZCCTb* genes found at the *HvVRN2* locus in barley, daylength is the major environmental cue regulating gene expression in vernalization-responsive winter barley (cv Sonja). Expression of both genes was high in long days and low in short days (Fig. 3B). Vernalization affected expression of only the *HvZCCTb* gene, and only when plants were grown under long days (Fig. 7B). The effect of vernalization on *HvZCCTb* expression was much slower than that of daylength (Figs. 4A and 7B). On the basis of this expression pattern, we predict that *HvVRN2* (*HvZCCTa* and *HvZCCTb*) operates as a repressor of flowering only under long-day conditions. It has recently been shown that *HvVRN2* genotype influences flowering time predominantly in long-day conditions (Karsai et al., 2005). The observation that *HvZCCTa* and *HvZCCTb* are expressed only in long-day-grown plants explains why the *HvVRN2* genotype affects flowering only in long days. Daylength regulation of *HvZCCTa* and *HvZCCTb* expression may account for why variation in the *HvVRN2* genotype does not always affect flowering time in populations of field-grown barley plants (e.g. Laurie et al., 1995).

**Figure 6.** Vernalization does not block daylength induction of *HvZCCTa* or *HvZCCTb*. Semiquantitative RT-PCR using gene-specific primers was used to compare *HvZCCTa* or *HvZCCTb* expression in plants that have been vernalized for 9 weeks under SD and then returned to glasshouse conditions for 14 d under either SD (V) or LD (V + LD). Plants grown under glasshouse conditions for an equivalent length of time (98 SD) are shown as a developmental control. Expression was also assayed in nonvernalized plants grown under SD for 21 d and then shifted to LD for 2 weeks. Expression of *HvZCCTa* and *HvZCCTb* in plants maintained in SD (35 SD) are shown as a developmental control. Expression of *ACTIN* is shown as a loading comparison for all samples. LD, Long day; SD, short day.

causing strong induction of *HvVRN1* expression. Expression of *HvVRN1* increased further after vernalized plants were returned to glasshouse temperatures. This might imply autoregulation of *HvVRN1*, such that once *HvVRN1* is expressed beyond a certain threshold, it causes further activation of its own expression (Fig. 1E). Expression of *HvVRN1* also increased slowly during development in plants that had not been vernalized (Fig. 1A). Daylength conditions did not affect induction of *HvVRN1* by vernalization or *HvVRN1* expression postvernalization, but did affect the degree to which *HvVRN1* expression increased during development, with *HvVRN1* expression occurring earlier when plants were grown under long days (Fig. 1A).

Expression of *HvVRN1* was not affected by daylength in most spring varieties examined. Daylength activation of *HvVRN1* expression occurred in only one of the seven spring barleys examined (Fig. 2). The daylength response of *HvVRN1* expression seen in Icheon Naked and reported for Dicktoo (Danylik et al., 2003) may be due to specific combinations of genotypes at photoperiod and vernalization loci. Both Dicktoo and Icheon Naked are photoperiod responsive and both have the *HvVRN2* spring habit genotype (*Hvvrn2*). The combination of a strong photoperiod response and the absence of *HvVRN2* function may be a prerequisite for induction of *HvVRN1* by long days. Alternatively, the daylength induction of *HvVRN1* in these varieties may be a property of the *HvVRN1* alleles that they carry.

For the *HvZCCTa* and *HvZCCTb* genes found at the *HvVRN2* locus in barley, daylength is the major environmental cue regulating gene expression in vernalization-responsive winter barley (cv Sonja). Expression of both genes was high in long days and low in short days (Fig. 3B). Vernalization affected expression of only the *HvZCCTb* gene, and only when plants were grown under long days (Fig. 7B). The effect of vernalization on *HvZCCTb* expression was much slower than that of daylength (Figs. 4A and 7B). On the basis of this expression pattern, we predict that *HvVRN2* (*HvZCCTa* and *HvZCCTb*) operates as a repressor of flowering only under long-day conditions. It has recently been shown that *HvVRN2* genotype influences flowering time predominantly in long-day conditions (Karsai et al., 2005). The observation that *HvZCCTa* and *HvZCCTb* are expressed only in long-day-grown plants explains why the *HvVRN2* genotype affects flowering only in long days. Daylength regulation of *HvZCCTa* and *HvZCCTb* expression may account for why variation in the *HvVRN2* genotype does not always affect flowering time in populations of field-grown barley plants (e.g. Laurie et al., 1995).

**Figure 7.** RT-PCR analysis of *HvZCCTa* and *HvZCCTb* expression during vernalization under long-day conditions. A, Real-time RT-PCR quantification of *HvZCCTa* and *HvZCCTb* expression, relative to *ACTIN*, in vernalized and nonvernalized plants. Plants (cv Sonja; *Hvvrn1/Hvvrn2*) were germinated and grown in long days. After 21 d, plants were placed at vernalizing temperatures in long days for 9 weeks (+V) or remained at normal glasshouse temperatures (–V) for the same length of time. The indicated error is the SD for three technical repeats. B, *HvZCCTa* and *HvZCCTb* expression levels were assayed separately using gene-specific primers at a number of points during the vernalization treatment (1, 3, 5, 7, and 9 weeks). Expression levels are compared to those in nonvernalized control plants harvested at identical time points. Expression of *ACTIN* is shown as a loading comparison for all samples.
It has been proposed that VRN2 acts to repress VRN1, and that a decrease in VRN2 expression during vernalization leads to induction of VRN1 (Yan et al., 2004b). According to this model, expression of VRN1 should occur when VRN2 is not expressed. We found that this is not always the case. Growing plants in short-day conditions represses HvVRN2 (HvZCCTa and HvZCCTb) expression, but there is no increase in HvVRN1 expression (Figs. 1C and 6). We also found that HvVRN1 is expressed despite HvVRN2 (HvZCCTa and HvZCCTb) expression when plants are grown for extended periods in long days (Figs. 1A and 7B), or when plants vernalized under short days were shifted to long-day conditions at greenhouse temperatures. Plants subjected to these treatments do flower, showing that expression of HvVRN1 is able to overcome the repression of flowering mediated by HvVRN2.

In the Sloop × Halcyon doubled haploid population, expression of HvVRN1 in lines carrying a spring HvVRN1 allele represses expression of HvZCCTa and HvZCCTb. A similar effect was observed in both diploid and hexaploid wheat lines carrying spring alleles of VRN1 (Loukoianov et al., 2005). If HvVRN1 is able to repress expression of HvZCCTa and HvZCCTb, then it is possible that, during vernalization under long days, induction of HvVRN1 represses the HvZCCTb gene, rather than the cold treatment per se. However, there is not a strict correlation between expression of HvVRN1 and HvZCCTa or HvZCCTb in barley. Repression of HvZCCTa and HvZCCTb by HvVRN1 may depend upon the level of HvVRN1 expression. In hexaploid wheat, spring alleles of VRN1 that confer different levels of VRN1 expression were found to repress VRN2 expression to different extents (Loukoianov et al., 2005). Alternatively, the effect of HvVRN1 on HvVRN2 (HvZCCTa and HvZCCTb) may be dependent upon interactions with other genes, which are not uniformly expressed in the treatments we have examined.

Our data are difficult to reconcile with the current model of the vernalization response pathway. We suggest an alternative model to explain interactions between VRN1 and VRN2 (Fig. 9). We propose that flowering in winter cereals can be triggered by vernalization, daylength, or developmentally regulated (autonomous) pathways, as is the case in Arabidopsis (Arabidopsis thaliana; see Mouradov et al., 2002), and that VRN1 and VRN2 may be regulated by different flowering-time pathways. In the vernalization pathway, induction of VRN1 by vernalization has a major role in determining the timing of flowering. In addition to this function in the vernalization response pathway, we suggest that VRN1 function is critical to the floral transition and that expression of VRN1 is activated by other flowering-time pathways such as a developmental pathway during extended growth in long-day conditions. We suggest that VRN2 operates in a daylength response pathway to counteract induction of flowering by long days and prevent precocious flowering in plants that have not been vernalized. When VRN2 function is lost, plants develop more rapidly toward flowering under long-day conditions and this causes earlier activation of VRN1 expression. According to our model, expression of VRN1 can overcome the repressive effect of VRN2, possibly by down-regulating its expression.

VRN2, which is regulated primarily by daylength cues but also by vernalization, may play an important role in integrating daylength and vernalization pathways in winter cereals. This is different from Arabidopsis, where integration of daylength and vernalization pathways is mediated by regulatory interactions between a vernalization-responsive repressor of flowering, FLOWERING LOCUS C, and the daylength-responsive promoters of flowering, FLOWERING LOCUS T and SUPPRESSOR OF OVEREXPRESSION OF CO1 (Michaels et al., 2005).
vernalization response has apparently evolved separately in monocots and dicots, resulting in different regulatory interactions between the vernalization response pathway and other molecular pathways controlling the timing of flowering. Identification of regulatory targets of VRN2 will be important in understanding the control of flowering time in winter cereals.

*HoVRN1* expression is induced by vernalization under long days in barley varieties that carry deletions at the *HoVRN2* locus (e.g. Perga and Dunja; Trevaskis et al., 2003) and under short days when *HoVRN2* (*HvZCCTa* and *HvZCCTb*) is expressed at a low level. Regulators other than VRN2 may be required to induce VRN1 during vernalization. MADS-box genes are likely candidates for regulators of VRN1. There are at least three MADS-box genes other than VRN1 that respond to cold or daylength cues in winter cereals (Trevaskis et al., 2003; Kane et al., 2005) and the promoters of both VRN1 and VRN2 contain sequences that are potential MADS-box binding sites (Yan et al., 2003; Ay485644, 333,235–333,245 bp). The identification of such regulators of VRN1 will be an important step in further understanding the vernalization response in winter cereals.

**MATERIALS AND METHODS**

**Plant Growth**

Plants were grown in pots of soil in sunlit glasshouses under long days (16-h light/8-h dark, with supplementary lighting used when natural light levels dropped below 200 μE) or short days (8-h light/16-h dark). Plants were harvested at the middle of the light period for both treatments. Glasshouses had an average temperature of 19°C. For vernalization treatments, plants were maintained at an average temperature of 8°C under the same light regimes as outlined above for 9 weeks.

**RNA Extraction and Gel-Blot Analysis**

Total RNA was extracted from whole seedlings, excluding root tissue, using the method of Chang et al. (1993). Twenty micrograms of total RNA were separated in formaldehyde gels and then blotted onto Hybond-N membrane (Amersham Biosciences) as described in Ausubel et al. (1994). RNA blots were hybridized with riboprobes and washed using the protocol of Dellerus et al. (1994). A *HoVRN1*-specific riboprobe was amplified from cDNA using the primers 5′-TTACATGGTAGATTACTCGTACAGCC-3′ and 5′-TGAAGCTCGAGAAATGGTTG-3′. A template to produce an antisense *HoVRN2* riboprobe was amplified from genomic DNA using the primers 5′-GCTAGAGCTACAATATCAATTG-3′ and 5′-GGACACCTTGGTTACAGG-3′. Riboprobes were synthesized using the Promega T7 in vitro transcription kit following the protocol provided. Blots were visualized with a phosphor imager for varying lengths of time, dependent on signal intensity.

**RT-PCR and Real-Time PCR Analysis**

An oligo(T) primer (T<sub><sup>5</sup></sub>′[G/C/A]) was used to prime first-strand cDNA synthesis from 5 μg of total RNA by using the SuperScript reverse transcriptase enzyme (Invitrogen) according to the manufacturer’s instructions. A single RT reaction was performed for each RNA sample. RT-PCR was performed using AmpliTaq DNA polymerase (Applied Biosystems) in the buffer supplied by the manufacturer with 1.5 mM MgCl<sub>2</sub>. Cycling conditions were 40 cycles (except for *ACTIN*, 25 cycles) of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. Fragments were visualized by agarose gel electrophoresis. Real-time PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma) in 20-μl reactions, consisting of 1× SYBR Green JumpStart Taq ReadyMix, 1 μM of each primer, and cDNA corresponding to 62.5 ng of total RNA. Reactions were run on a Rotor-Gene 2000 or 3000 Real-Time Cycler (Corbett Research). Cycling conditions were 10 min at 95°C, 40 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-s hold at each temperature). Fluorescence data were acquired at the 72°C step and during the melting-curve program. Three replicate reactions for each cDNA-primer combination were performed for each sample in the same run. For each cDNA sample, *ACTIN* levels were also quantified in the same run in three replicate reactions. Expression levels relative to *ACTIN* were then calculated using the ROTORGENE software package (Corbett Research), which compares reaction takeoff points (cycle number). Amplification efficiencies of each primer set are considered. A technical repeat is defined as quantification of one replicate reaction for a gene of interest relative to one *ACTIN* reaction from the same sample. Two biological repeats were carried out for each experiment. All experiments showed similar trends in separate biological repeats. For RT-PCR, primers were designed to be cDNA specific by flanking introns or crossing exon-exon boundaries. For *HoVRN1*, primers 5′-TGAGACCTGCAGAGTGTTG-3′ and 5′-TATAGCCGAGTCCATTAGGC-3′ were used for both RT-PCR and real-time PCR. Gene-specific primer pairs 5′-ATACACCATCAGGAACAC-3′/5′-CTCCGAATGAGCAGATG-3′ and 5′-ATACACCATCAGGAACATGC-3′/5′-CGCCGATAATGCCAGATG-3′, designed to the promoters of both *HoZCCTa* and *HoZCCTb*, were used. For *HoZCCTa*, primers 5′-AACATCATACATGACACAGAGCAG-3′ and 5′-AGCTTGGACGGCGCCCTG-3′, designed to sequence AY687931, were used for RT-PCR. As this primer pair is not cDNA specific, first-strand cDNA synthesis was primed from total RNA that had been Dnase treated using RQ1 RNase-Free DNase (Promega) according to the manufacturer’s instructions. Expression of a barley (*Hordeum vulgare*) *ACTIN* gene (AY145451) was used as a control to normalize for the amount of cDNA present in each sample. Primers 5′-CGGCGTCTTCTCTCTATG-3′ and 5′-GCTTCTCTCTTGATGCTCCCTA-3′ were used to amplify AY145451 for both RT-PCR and real-time PCR. Gene-specific primer pairs 5′-GCTATCAGTTGCTGCT-3′/5′-AACACCGGGAATGGT-3′, 5′-CCTCCTACCCACAGA-3′ and 5′-AGAACCGATGGAAGC-3′, designed to sequences for *HoZCCTa* and *HoZCCTb* respectively. Primers described in Fu et al. (2005) that discriminate between alleles with and without a deletion in the first intron were used for genotyping of *HoVRN1* in the Sloop × Halcyon population.
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LITERATURE CITED


