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RESEARCH PAPER

The influence of vernalization and daylength on expression of flowering-time genes in the shoot apex and leaves of barley (*Hordeum vulgare*).

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Abstract

Responses to prolonged low-temperature treatment of imbibed seeds (vernalization) were examined in barley (*Hordeum vulgare*). These occurred in two phases: the perception of prolonged cold, which occurred gradually at low temperatures, and the acceleration of reproductive development, which occurred after vernalization. Expression of the *VERNALIZATION1* gene (*HvVRN1*) increased gradually in germinating seedlings during vernalization, both at the shoot apex and in the developing leaves. This occurred in darkness, independently of *VERNALIZATION2* (*HvVRN2*), consistent with the hypothesis that expression of *HvVRN1* is induced by prolonged cold independently of daylength flowering-response pathways. After vernalization, expression of *HvVRN1* was maintained in the shoot apex and leaves. This was associated with accelerated inflorescence initiation and with down-regulation of *HvVRN2* in the leaves. The largest determinant of *HvVRN1* expression levels in vernalized plants was the length of seed vernalization treatment. Daylength did not influence *HvVRN1* expression levels in shoot apices and typically did not affect expression in leaves. In the leaves of plants that had experienced a saturating seed vernalization treatment, expression of *HvVRN1* was higher in long days, however. *HvFT1* was expressed in the leaves of these plants in long days, which might account for the elevated *HvVRN1* expression. Long-day up-regulation of *HvVRN1* was not required for inflorescence initiation, but might accelerate subsequent stages of inflorescence development. Similar responses to seed vernalization were also observed in wheat (*Triticum aestivum*). These data support the hypothesis that *VRN1* is induced by cold during winter to promote spring flowering in vernalization-responsive cereals.

Key words: Barley, floral development, *FT*, MADS box gene, photoperiod, vernalization, *VRN1*, *VRT2*, *VRN2*, wheat.

Introduction

Prolonged cold treatment of imbibed seeds, or vernalization, promotes flowering of many temperate cereals, including varieties of wheat, barley, oat, and rye. When grown without cold pre-treatment, the same varieties grow vegetatively for extended periods and often fail to flower altogether. Thus, these cereal varieties have a '*Kaltbedurfnis*', or cold requirement, that must be met for rapid transition to reproductive growth (Gassner 1918). Although originally defined as prolonged cold treatment of seeds, vernalization also accelerates flowering when applied to plants during the vegetative growth phase (Gott, 1957; Flood and Halloran, 1984).

Flowering of temperate cereals is also accelerated by long days (see Purvis, 1934). Studies in *Arabidopsis* have shown

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that the long-day flowering response is controlled by *FLOWERING LOCUS T* (*FT*) (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). The FT protein is produced in leaves in long days and transported to the shoot apex to promote reproductive development (Corbesier *et al.*, 2007). *FT-like 1* (*TaFT1* in wheat or *HvFT1* in barley) is an orthologue of *FT* likely to fulfil a similar role in temperate cereals (Turner *et al.*, 2005).

The requirement for vernalization suppresses the long-day flowering response (Hemming *et al.*, 2008). This ensures that flowering is delayed before winter, reducing the risk of frost damage (Mahfoozi *et al.*, 2001; Limin and Fowler, 2006). Many varieties flower rapidly without vernalization, however, and this natural variation in vernalization requirement has been useful to adapt varieties to warmer growing conditions. Genes controlling natural variation in vernalization requirement have been identified in temperate cereals: *VERNALIZATION1* (*VRN1*), *VRN2*, and *VRN3* (reviewed in Trevaskis *et al.*, 2007*a*; Distalfeld *et al.*, 2009).

VRN1 is a *FRUITFULL*-like MADS box transcription factor that is essential for flowering in temperate cereals (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003; Preston and Kellog, 2006; Shitsukawa *et al.*, 2007). In varieties that require vernalization, *VRN1* is activated by prolonged exposure to low temperatures (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003). In other varieties, *VRN1* is expressed without vernalization, reducing or removing the requirement for vernalization (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003). Mutations in the promoter or deletions within the first intron of the *VRN1* gene are associated with increased *VRN1* expression in these varieties (Yan *et al.*, 2003; Fu *et al.*, 2005).

VRN2, or *HvVRN2* in barley, is a floral repressor (Takahashi and Yasuda, 1971; Dubcovsky *et al.*, 1998; Yan *et al.*, 2004). *VRN2* is active in long days (Karsai *et al.*, 2005; Dubcovsky *et al.*, 2006; Trevaskis *et al.*, 2006), where it represses *FT1* to delay the long-day flowering response until plants are vernalized (Hemming *et al.*, 2008). *VRN1* represses expression of *VRN2* (Loukoianov *et al.*, 2005; Trevaskis *et al.*, 2006; Hemming *et al.*, 2008), so when plants are vernalized *VRN1* is likely to repress *VRN2* to allow long-day induction of *FT1* (Trevaskis *et al.*, 2007*a*; Hemming *et al.*, 2008). Loss-of-function mutations at the *VRN2* locus allow expression of *FT1* without prior vernalization, causing rapid flowering in long days (Yan *et al.*, 2004; Karsai *et al.*, 2005; Hemming *et al.*, 2008). This requires an active *PHOTOPERIOD1* gene (Hemming *et al.*, 2008), which promotes long-day induction of *HvFT1* (Turner *et al.*, 2005). Similarly, dominant alleles of *VRN3* elevate *FT1* expression levels to accelerate flowering and bypass the vernalization requirement (Yan *et al.*, 2006; Faure *et al.*, 2007). *VRN3* has been mapped to the *FT1* sequence, suggesting that *FT1* is the *VRN3* gene (Yan *et al.*, 2006). Dominant *VRN3* alleles in wheat are associated with an insertion in the promoter of the *TaFT1* gene (Yan *et al.*, 2006) and, although there is an association between polymorphisms in the *HvFT1* gene and dominant alleles of *VRN3* in barley (Yan *et al.*, 2006), the molecular basis for the increased expression of *HvFT1* associated with dominant *VRN3* alleles in barley is not clear (Hemming *et al.*, 2008; Stracke *et al.*, 2009).

In barley plants that lack *HvVRN2*, expression of *HvVRN1* is induced in long days without prior cold treatment (Yan *et al.*, 2003; Trevaskis *et al.*, 2006; Hemming *et al.*, 2008). *HvVRN1* is also expressed without cold treatment in barleys plants with dominant alleles of *VRN3* (Yan *et al.*, 2006). In these genotypes, *HvFT1* is expressed without prior cold treatment and the FT1 protein might activate *VRN1* through interactions with FD-like proteins (Li and Dubcovsky, 2008). It is unclear whether this is a feature of vernalization-induced flowering, where low-temperature induction of *VRN1* precedes long days (Hemming *et al.*, 2008). Furthermore, the potential for low-temperature and long-day activation of *VRN1* complicates analysis of the vernalization response in cereals. For example, although expression of *VRN1* increases in leaves when wheat plants are vernalized in long days (Yan *et al.*, 2003; Preston and Kellog 2008), it is unclear whether this is a response to low temperatures or to the combined effects of low temperatures and long days. Analysis of gene expression in plants vernalized in short days has been useful to determine the discrete effects of low temperature on expression of the vernalization genes in cereals (von Zitzewitz *et al.*, 2005; Dubcovsky *et al.*, 2006; Trevaskis *et al.*, 2006; Fu *et al.*, 2007; Hemming *et al.*, 2008), but such studies have not yet examined responses to cold in specific organs.

The seed vernalization response first investigated by Gassner (1918) provides an ideal experimental system to resolve the effects of low temperature and daylength cues on expression of flowering-time genes during vernalization-induced flowering. Seeds can be exposed to long cold treatments in darkness then sown in different daylengths. In this study the molecular responses to seed vernalization are examined by assaying the expression of flowering-time genes in different organs during and after seed vernalization.

Materials and methods

Seeds of the winter barley (*Hordeum vulgare*) variety cv. Sonja, or the winter wheat cv. Norstar, were imbibed, planted in foil-covered pots, and exposed to cold treatments of different durations (e.g. 0, 1, 2, 3, 4, 5, 7, and 9 weeks) at 2 ± 1 °C. After 9 weeks in these conditions, plants reached an average coleoptile length of 4 cm. At each time point, embryos or germinating seedlings were harvested for RNA extraction and apex dissection. The developing roots and leaves were also isolated after 4 weeks cold treatment. Following cold treatment, seedlings were transferred to glasshouses (temperature at 18 ± 2 °C) and grown in either short (8 h light/16 h dark) or long (16 h light/8 h dark) days. Supplementary light was provided when natural levels dropped below 200 μE. Plants were harvested at the third leaf stage (Z=13,21, Zadoks *et al.*, 1974), ~2 weeks after the end of the cold treatment, and leaves and shoot apices were harvested for RNA extraction. Final leaf number

(FLN), and days to heading were recorded for each treatment. Similarly, seeds of a barley which lacks *HvVRN2* (Line 347, *ΔHvVRN2/HvVRN1/PPD-H1*; Hemming *et al.* 2008) were imbibed and exposed to cold for 9 weeks, then transferred to glasshouse temperatures in short days. Plants were harvested for apex dissection and RNA extraction after 9 weeks of cold treatment, and from glasshouses at the third leaf stage, ~2 weeks after the end of the cold treatment.

Apex dissection and flowering time measurements

Apices were isolated under a binocular dissecting microscope and then digitally photographed on a Leica M8 digital camera. Apex samples included the apex, the base of the apex, and any leaf primordia <0.2 mm in length. Leaves were numbered sequentially and plants were grown until the flag leaf emerged to determine FLN. Heading date was measured as the day when the head first emerged from the sheath on the main shoot. FLN and heading date were measured for 9–18 plants for each data point. Average FLN and days to heading are presented, and error bars show the standard error (SE).

Gene expression analysis

Total RNA was extracted using the method of Chang *et al.* (1993). An oligo(T) primer (T18[G/C/A]) was used to prime first-strand cDNA 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 reaction was performed for each RNA sample. Quantitative reverse transcription-PCR (qRT-PCR) was performed on a Rotor-Gene 3000 real-time cycler (Corbett Research). The primers used for *HvVRN1*, *HvVRN2* (*HvZCCTb*), *HvFT1*, *Barley MADS1* (*BM1*), *BM10*, *Hordeum vulgare VEGETATIVE to REPRODUCTIVE TRANSITION 2* (*HvVRT2*), and *ACTIN* have been described previously (Trevaskis *et al.*, 2006, 2007*b*). For analysis of *VRN1* and *VRN2* expression in wheat (cv. Norstar), the primer sets described above, which are predicted to amplify all three genomes from wheat, were used. The following primers were used for other MADS box genes: *BM3* 5′-GCCGTCACCAGCACAAGCAA-3′ and 5′-CCCCATTCACCCTGTAGCAAAGA-3′; *BM7* 5′-GCTTGACCAGATAGAGAACCAAATAG-3′ and 5′-GCTGGTGGTGGTGGTGTCTTGC3′; *BM8* 5′-CGCACAGCAGCCGACACCTA-3′ and 5′-TGCCTTTGGGGGAGAAGACG-3′; and *BM9* 5′-TCGTCCTTGAAGCACATTAGAAC-3′ and 5′-GGGTTCACCAGCAGCATCAAGGG-3′. Primer pairs amplify cDNA-specific DNA products. qRT-PCR was performed using Platinum *Taq* DNA polymerase (Invitrogen). Cycling conditions were 4 min at 94 °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 (72–95 °C with a 5 s hold at each temperature). Fluorescence data were acquired at the 72 °C step and during the melting curve program. Expression levels of genes of interest were calculated relative to *ACTIN* using the comparative quantification analysis method (Rotogene-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 quadruplicate, 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 four RT-PCR quantifications (gene of interest versus *ACTIN*) for each RNA sample; each sample was extracted from 30–50 apices, 3–5 leaves or 3–5 seedlings. Similar results were obtained in a second set of samples from the same experiments. Where Student's *t*-tests show that expression levels for a gene of interest differed significantly between two treatments (Figs 2F and 6A, B), differences of similar significance levels were found in both sample sets.

Results

Prolonged cold treatment of imbibed seeds promotes flowering by accelerating inflorescence initiation

Flowering time, as indicated by FLN and days to heading, was assayed in plants grown from seeds germinated with or without cold treatment. In short days, control plants (no cold treatment) did not flower within 150 d (FLN >25), but plants grown from seeds that had experienced >4 weeks of cold during germination flowered within 130 d (FLN=14). Longer cold treatments caused no further reduction in FLN in short days and little further reduction in the days to heading (Fig. 1A, B).

In long days, control plants (no cold treatment) flowered within 120 d (FLN=20). Plants grown from cold-treated seeds flowered earlier than control plants. Short-term cold treatments accelerated flowering. One week cold treatment reduced FLN to 18, for example, and longer cold treatments further reduced flowering time and FLN. The 9 week cold treatment had the greatest impact on flowering time, reducing the time taken to flower to 47 d (FLN=10) (Fig. 1A, B). Beyond 9 weeks, longer cold treatments had no additional impact on flowering time or FLN (FLN=10 for plants subjected to 11 weeks cold treatment). Thus, 9 weeks cold treatment of seeds completely fulfils the vernalization requirement of this barley (vernalization saturation point).

Cold treatment of imbibed seeds accelerated flowering by promoting early stages of shoot apex development (Fig. 1C), consistent with the reduced FLN of plants grown from cold-treated seeds. The shoot apex remained vegetative at the end of even the longest vernalization treatments (Fig. 1C), so accelerated floral development occurred after vernalization during growth at normal glasshouse temperatures. Shoot apex development was accelerated to a greater extent when vernalized plants were grown in long days (Fig. 1A, C). Stem elongation and post-initiation (double ridge stage, when floral primordia develop at the shoot apex) inflorescence development also occurred rapidly when vernalized plants were grown in long days (Fig. 1C). In

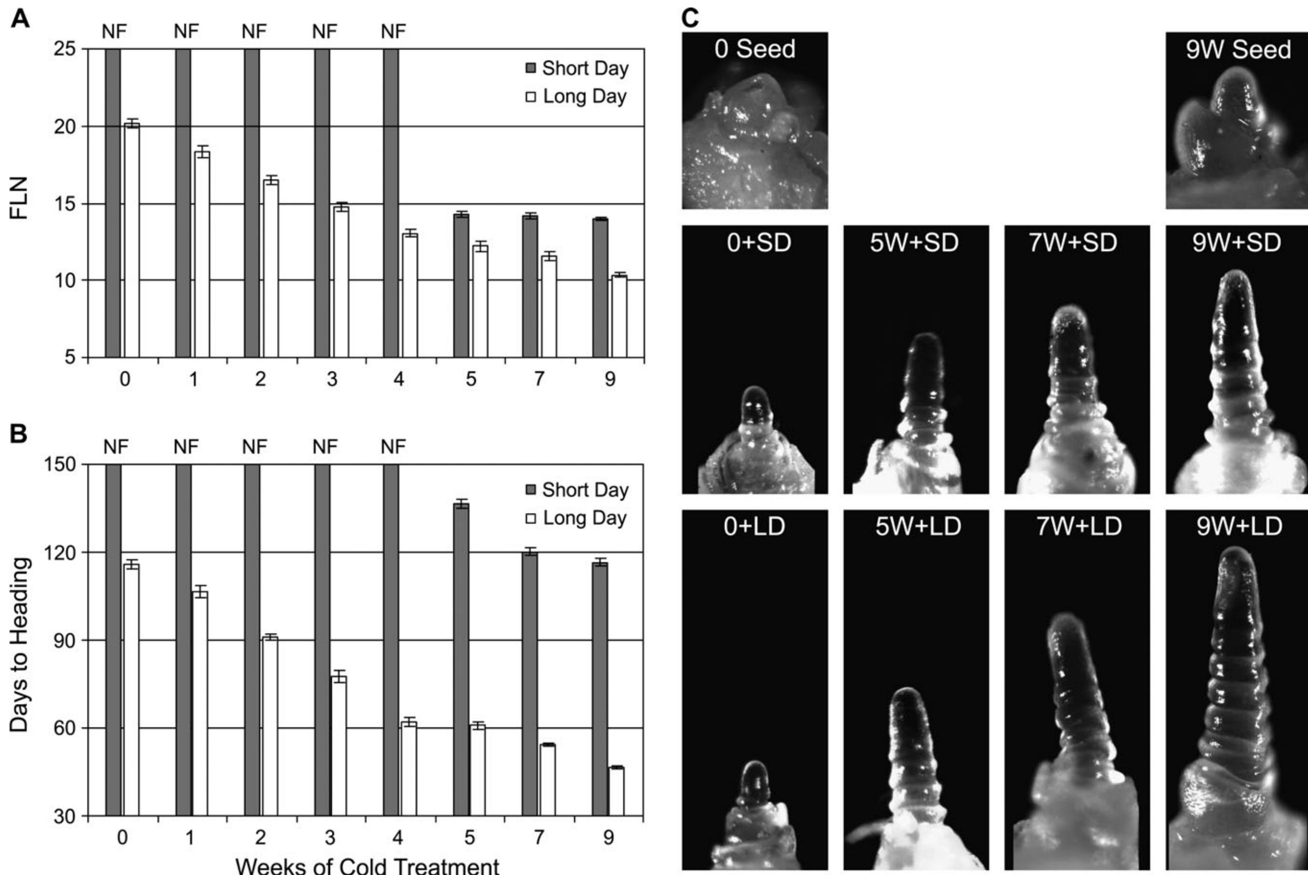

Fig. 1. The effect of seed vernalization treatment on flowering time of a winter barley. (A) Final leaf number (FLN), in short or long days, of non-vernalized (0) plants of a winter barley (cv. Sonja) versus plants subjected to seed vernalization treatments of different durations (1–9 weeks). NF denotes no flowering observed. (B) Days to flowering (heading) for the same treatments. (C) Images of representative shoot apices (50× magnification) from: imbibed seeds (0 seed), cold-treated seeds (9W seed), and plants at the third leaf stage in either short day or long day conditions (+SD or +LD), ~2 weeks after the end of seed vernalization treatments of different durations (0, 5, 7, or 9 weeks). For example, 5W+SD refers to plants that were vernalized for 5 weeks in darkness then grown in short days.

short days, the shoot apex elongated more than in control plants and the apex developed to the double ridge stage, but further inflorescence development and stem elongation occurred slowly. Consequently flowering (head emergence) was delayed in short days in comparison with plants grown in long days, after equivalent vernalization treatment.

Cold treatment induces expression of HvVRN1 *in germinating seeds*

Expression of *HvVRN1* was assayed during germination at low temperatures (seed vernalization). *HvVRN1* expression was first detected after 4 weeks cold treatment and was induced to higher levels with longer cold treatments (Fig. 2A). Expression of *HvVRN1* increased in the developing leaves, roots, and the shoot apex (Fig. 2B). Elevated *HvVRN1* transcript levels were maintained during subsequent development at normal glasshouse temperatures. At the third leaf stage, ~2 weeks after the end of cold treatment, *HvVRN1* was expressed in both shoot apices and leaves (Fig. 2C, D). In both tissue types, *HvVRN1* transcript levels correlated with the length of cold treatment applied during germination (Fig. 2C, D).

Daylength did not influence expression of *HvVRN1* in the shoot apex of vernalized plants (Fig. 2E). Similarly, daylength did not influence expression of *HvVRN1* in the leaves of plants grown from seeds vernalized for 5 or 7 weeks. In plants grown from seeds subjected to 9 weeks of seed vernalization, expression of *HvVRN1* was ~2-fold higher in the leaves in long days than in short days (Fig. 2F).

HvVRN2 *is down-regulated in the leaves of vernalized plants but is not required for the low-temperature flowering response*

Expression of the floral repressor *HvVRN2* was not detected in seeds germinating in the dark, regardless of temperature (Fig. 3A). *HvVRN2* was expressed in the leaves of plants in long days (Fig. 3B) and, although *HvVRN2* expression did not change during vernalization, expression of *HvVRN2* was lower in the leaves of plants grown from cold-treated seeds. The extent to which *HvVRN2* was down-regulated correlated with the length of cold treatment applied (Fig. 3C). There was an inverse relationship between *HvVRN1* and *HvVRN2* expression levels in leaves in plants grown in long days (compare Figs 2C and 3C), consistent with the

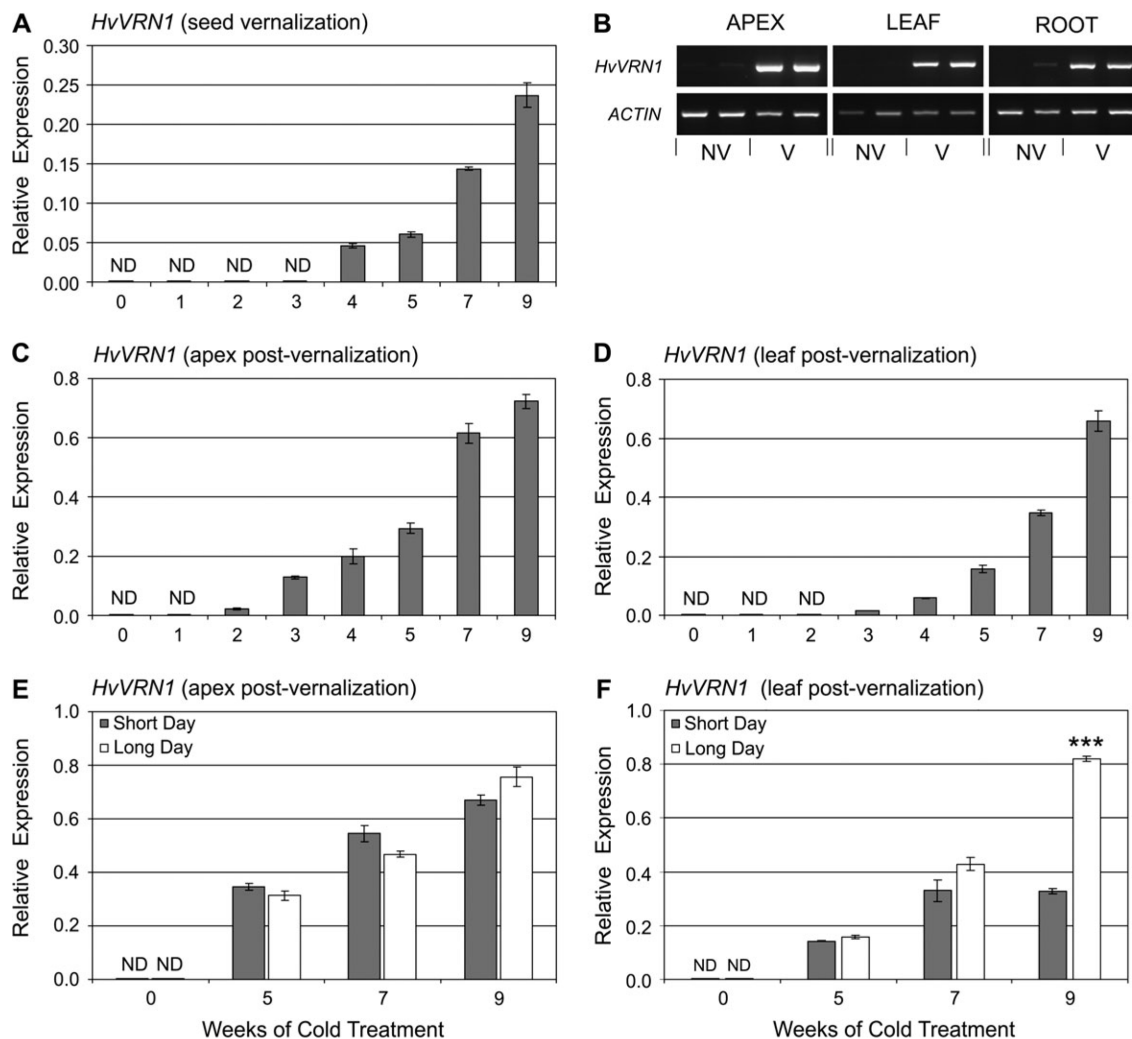

Fig. 2. The effect of seed vernalization treatment on expression of *HvVRN1*. (A) Expression of *HvVRN1* in seedlings at different time points (weeks) during seed vernalization. (B) RT-PCR analysis of *HvVRN1* expression (35 cycles) in different organs during seed vernalization. Expression of *ACTIN* (25 cycles) is shown as a positive control, and two biological repeats are shown for each data point. NV refers to samples from non-vernalized plants and V refers to samples from vernalized plants. (C) Expression of *HvVRN1* in the shoot apex at the third leaf stage, in non-vernalized plants (0) compared with plants that received seed vernalization treatments of different durations (1–9 weeks). (D) Expression of *HvVRN1* in leaves (fully expanded second leaf) at the third leaf stage in the same experiment. (E) *HvVRN1* expression in shoot apex in short versus long days at the third leaf stage, in non-vernalized plants (0) or after seed vernalization treatments of different durations (5, 7, or 9 weeks). (F) Comparison of *HvVRN1* expression in leaves (fully expanded second leaf) in the same experiment. Expression of *HvVRN1* was assayed by quantitative RT-PCR and is shown relative to *ACTIN*. ND denotes no expression detected. Asterisks indicate *P*-values of Student's *t*-test: ****P* <0.001.

suggestion that *HvVRN1* down-regulates *HvVRN2* in vernalized plants (Trevaskis *et al.*, 2006, 2007*a*). This relationship was not observed in short days, where *HvVRN2* was expressed at uniformly low levels in leaves irrespective of cold treatment or *HvVRN1* expression levels (Fig. 3D).

The influence of seed vernalization on *HvVRN1* transcript levels and shoot apex development was assayed in a barley which lacks *HvVRN2*. In this barley, expression of *HvVRN1* was low during germination but was induced by prolonged cold treatment (Fig. 4A). Cold treatment also accelerated inflorescence initiation during subsequent development at normal glasshouse temperatures, and at the third leaf stage the shoot apex of cold-treated plants had progressed to the double ridge stage whereas the apices of control plants remained vegetative (Fig. 4B).

Seed vernalization allows long-day induction of HvFT1 *in leaves*

Expression of *HvFT1* was assayed during and after seed vernalization treatments of different durations and in non-vernalized control plants at the same stage of development. *HvFT1* expression was not detected in seedlings germinating in darkness, regardless of temperature. Following germination, at the third leaf stage, *HvFT1* was not expressed in plants grown from seeds germinated without cold treatment

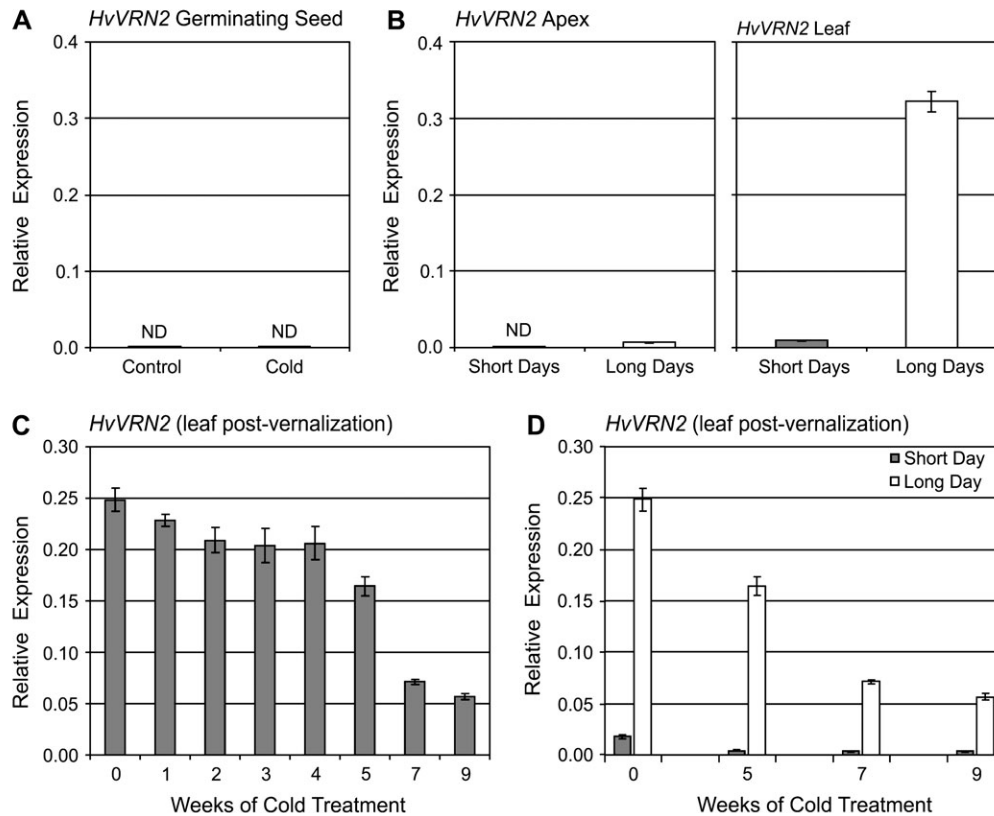

Fig. 3. The effect of seed vernalization treatment on *HvVRN2* expression. (A) *HvVRN2* expression in non-vernalized seedlings (control) versus seedlings germinated at low temperature for 7 weeks (cold). (B) Expression of *HvVRN2* in the shoot apex or second leaf for non-vernalized plants at the third leaf stage, in short versus long days. (C) Relative expression of *HvVRN2* in leaves (fully expanded second leaf) at the third leaf stage in long days, in non-vernalized plants (0), or after seed vernalization for different durations (1–9 weeks). (D) Relative expression levels of *HvVRN2* in leaves (fully expanded second leaf) from plants at the third leaf stage in short or long days. The comparison shows non-vernalized plants (0) versus plants grown from seeds vernalized for 5, 7, or 9 weeks. Expression of *HvVRN2* was assayed by quantitative RT-PCR and is shown relative to *ACTIN*. ND denotes no expression detected.

but was expressed in the leaves of plants grown in long days from seeds that were vernalized for 9 weeks (Fig. 5). No expression of *HvFT1* was detected in plants grown in short days, as has been reported previously for this barley variety (Hemming *et al.*, 2008).

The influence of low-temperature treatment on expression of other MADS box genes in barley

It has been suggested that the *SHORT VEGETATIVE PHASE-like* gene *HvVRT2* is repressed by cold and that this allows expression of *HvVRN1* to increase during vernalization (Kane *et al.*, 2005). Expression of *HvVRT2* increased slightly in vernalized seeds, as did the related gene *BM10* (Fig. 6A, B). Transcript levels of a third *SVP*-like MADS box gene, *BM1*, did not change during seed vernalization (Fig. 6C).

In addition to *HvVRN1*, there are two other *FRUIT-FULL*-like MADS box genes in barley: *BM3* and *BM8*. Expression of these genes was assayed before and after seed vernalization to determine whether these genes play a similar role to *HvVRN1* in the low-temperature flowering response. No expression of either *BM3* or *BM8* was detected in germinating seeds, and neither gene was activated by low-temperature treatment (Supplementary Fig. S1 available at *JXB* online). Similarly, no expression of the *SEPALLATA*-like MADS box genes *BM7* and *BM9* (Schmitz *et al.*, 2000) was detected in germinating seeds irrespective of temperature (Supplementary Fig. S1).

The seed vernalization response is similar in wheat and barley.

To determine whether low temperature and long day cues regulate expression of flowering-time genes in a similar manner in other temperate cereals, expression of *VRN1* and *VRN2* was examined in the winter wheat cultivar Norstar during and after seed vernalization treatments. For these experiments, primers predicted to amplify all three copies (A, B, and D genomes) of the target genes were used, to assay the sum expression levels of *VRN1* or *VRN2*. Seed vernalization promoted flowering in this wheat (Supplementary Fig. S2 at *JXB* online). Prolonged cold treatment induced expression of *VRN1* in imbibed wheat seeds, and expression of *VRN1* was maintained in the shoot apex and leaves of wheat plants after vernalization treatment (Fig.

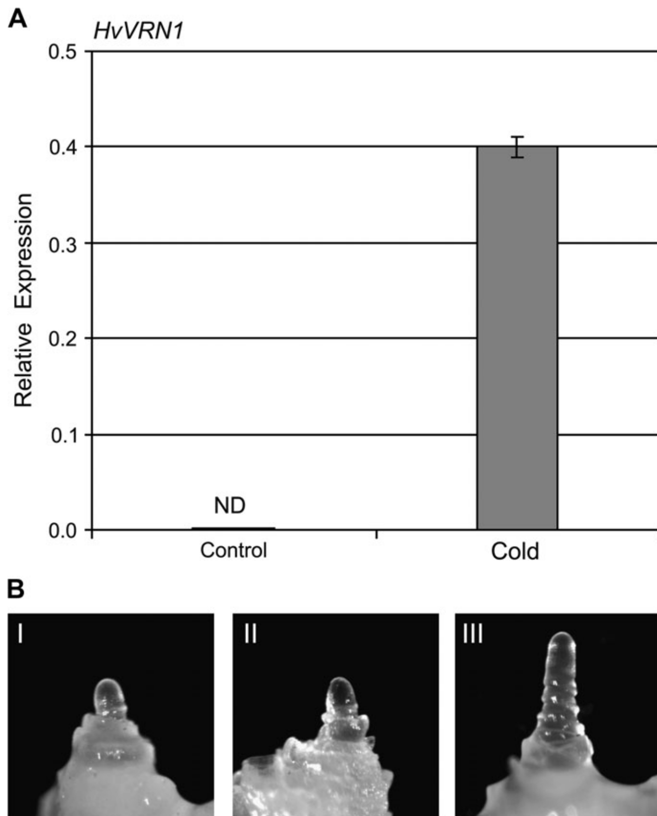

Fig. 4. The response to seed vernalization in a barley that lacks *HvVRN2*. (A) Expression of *HvVRN1* in seedlings of barley lacking *HvVRN2* (*HvVRN1*, *ΔHvVRN2/PPD-H1*), exposed to low-temperature treatment for 9 weeks during germination (cold) versus seedlings at the same stage of development that were germinated at normal growth temperatures (control). Expression of *HvVRN1* was assayed by quantitative RT-PCR and is shown relative to *ACTIN*. (B) The morphology of the shoot apex after 9 weeks of seed vernalization treatment (I), at the third leaf stage in either non-vernalized plants (II) or plants grown from seeds that were vernalized for 9 weeks (III) (×50 magnification). As this barley lacks *HvVRN2*, plants were grown in short days to prevent activation of flowering by the long-day response.

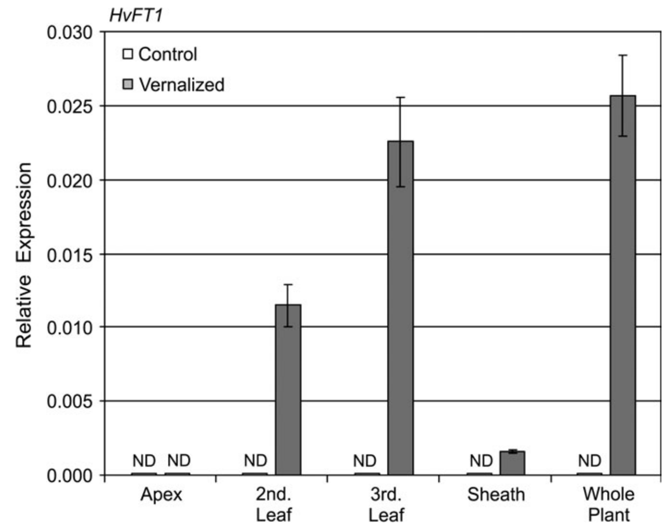

Fig. 5. The effect of seed vernalization treatment on expression of *HvFT1*. (A) Plants subjected to different vernalization treatments were grown in long days until the third leaf stage when different organs were isolated for gene expression analysis. Expression of *HvFT1* was assayed in the apex, second leaf, third leaf, or the sheath and compared with expression in whole plants. The comparison shows *HvFT1* expression in control (non-vernalized) versus vernalized plants (9 weeks seed vernalization). Expression of *HvFT1* was assayed by quantitative RT-PCR and is shown relative to *ACTIN*. ND denotes no expression detected.

7A–C). *VRN2* was expressed in leaves in long days, but in vernalized plants expression of *VRN2* in the leaves was lower than in control plants that had not experienced cold (Fig. 7D). The extent to which *VRN2* was down-regulated in the leaves of vernalized plants correlated with the length of cold treatment during germination (Fig. 7D). Thus, the molecular responses to seed vernalization appear to be similar in barley and wheat.

Discussion

The molecular responses to seed vernalization parallel those observed in plants vernalized in short days; *HvVRN1* is activated by low temperatures to a degree that correlates with the length of cold treatment, while *HvVRN2* and *HvFT1* are not expressed (Fig. 2; von Zitzewitz *et al.*, 2005; Trevaskis *et al.*, 2006; Hemming *et al.*, 2008). This suggests that the pathways controlling perception of cold in germinating seeds are similar to those in growing plants, and support the hypothesis that activation of *VRN1* mediates the low-temperature flowering response in cereals (see Trevaskis *et al.*, 2007*a*).

Compared with previous studies using plants vernalized in short days (von Zitzewitz *et al.*, 2005; Trevaskis *et al.*, 2006), analysis of the vernalization response in seeds has further clarified how *HvVRN1* is regulated. Induction of *HvVRN1* in seeds occurred in darkness, demonstrating that a low-temperature response pathway can activate expression of *HvVRN1* independently of light or daylength. This pathway is unlikely to involve *HvVRN2*, which is not expressed in seeds during vernalization (Fig. 3) and is not required for low-temperature induction of *HvVRN1* (Hemming *et al.*, 2008; Fig. 4). Similarly, induction of *HvVRN1* is unlikely to involve *HvFT1*, which is not expressed in seeds during vernalization. Low-temperature induction of *HvVRN1* is not limited to any particular organ type in barley seeds/seedlings (Fig. 2B).

Low-temperature induction of *HvVRN1* at the shoot apex precedes changes in shoot apex morphology (Fig. 1C), and so cannot be a consequence of floral development. Similar observations have been made by Yan *et al.* (2003) in einkorn wheat plants (*Triticum monococcum*) vernalized in long days. Preston and Kellog (2008) found that in oat (*Avena sativa*) induction of *VRN1* at the shoot apex occurs after vernalization, at the same time as floral

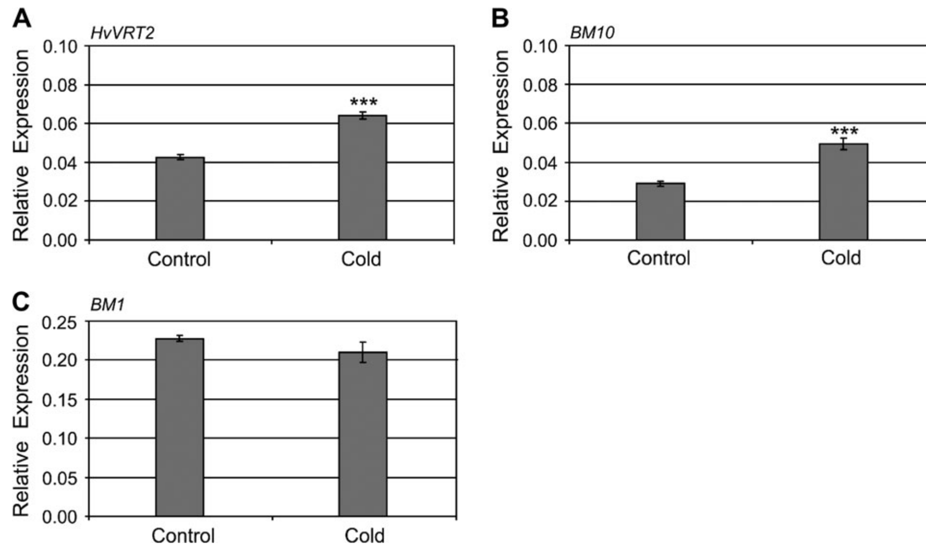

Fig. 6. The influence of seed vernalization on the transcript levels of other MADS box genes in barley. Expression of MADS box genes was assayed in seedlings at the 7 week vernalization time point (cold), and compared with non-vernalized plants at a similar stage of development (control). Expression of *HvVRT2* (A), *BM10* (B), and *BM1* (C) was assayed by quantitative RT-PCR and is shown relative to *ACTIN*. Asterisks indicate *P*-values of Student's *t*-test: ***P <0.001.

development. These conflicting findings might reflect differences between oats versus barley and wheat, or might be due to the different sensitivities of the gene expression analysis techniques used; *in situ* hybridization (Preston and Kellog, 2008) versus qRT-PCR (Yan *et al.*, 2003; this study).

Unlike *HvVRN1*, two related *AP1/FRUITFULL*-like genes, *BM3* and *BM8*, were not activated by seed vernalization. So while the oat (*A. sativa)* orthologue of *BM8*, *AsFUL2*, is induced in leaves when plants are vernalized in long days (Preston and Kellog, 2008), this might not be a general feature of the vernalization response in cereals. Expression of *HvVRT2*, another MADS box gene which has been suggested to play a role in the low-temperature response (Kane *et al.*, 2005), increased slightly during seed vernalization (Fig. 6A). This is similar to previous observations in vernalized plants (Trevaskis *et al.*, 2007*b*), and it seems unlikely that *HvVRT2* mediates low-temperature induction of *HvVRN1* in the manner suggested.

Following vernalization, *HvVRN1* expression levels remained high in the shoot apex and leaves (Fig. 2). This was associated with more rapid inflorescence initiation (Fig. 1A, C), consistent with genetic data that show that expression of *HvVRN1* promotes inflorescence initiation (Hemming *et al.*, 2008). This might be a specific consequence of elevated *HvVRN1* levels in the shoot apex. Expression of *HvVRN1* is also associated with reduced expression of *HvVRN2* and with induction of *HvFT1* in long days (Hemming *et al.*, 2008). Here it has been shown that down-regulation of *HvVRN2* occurs in leaves, and is a quantitative response; longer cold treatments are associated with greater down-regulation of *HvVRN2*. These findings have also been extended to wheat (Fig. 7). Overall, these data are consistent with the proposed role of *VRN1* in repressing *VRN2* to activate the long-day flowering response in leaves (Trevaskis *et al.*, 2007a).

The main determinant of *HvVRN1* expression levels in the leaves and shoot apices was the length of seed vernalization treatment (Fig. 2), similar to the response observed in whole plants (von Zitzewitz *et al.*, 2005; Trevaskis *et al.*, 2006; Hemming *et al.*, 2008). Daylength only influenced expression of *HvVRN1* in the leaves of plants grown from seeds vernalized for 9 weeks, where expression of *HvVRN1* was ~2-fold higher in long days (Fig. 2). This might be caused by elevated expression of *HvFT1* (Fig. 5), through interactions with FD-like proteins (Teper-Bamnolker and Samach, 2005; Li and Dubcovksy, 2008). Long-day induction of *HvVRN1* in leaves was not critical for inflorescence initiation, which occurred irrespective of daylength in vernalized plants, but might accelerate later stages of inflorescence development.

In general, the daylength flowering responses of vernalized barley plants are similar to those in rye (Purvis 1934); prolonged exposure to cold accelerates inflorescence initiation, but long days are required for rapid inflorescence development and for stem elongation. The present findings can explain the molecular events underlying these physiological responses; *HvVRN1* is induced by cold to accelerate inflorescence initiation, then long days induce *HvFT1* and further activate *HvVRN1* to accelerate subsequent stages of inflorescence development and stem elongation.

In summary, the seed vernalization treatment first described by Gassner (1918) provides a simple experimental system to analyse the vernalization response of cereals. Plants are vernalized in the dark, so artificial daylength conditions are not required during vernalization, and vernalized plants are easily matched to non-vernalized control plants at the same stage of development. Analysis

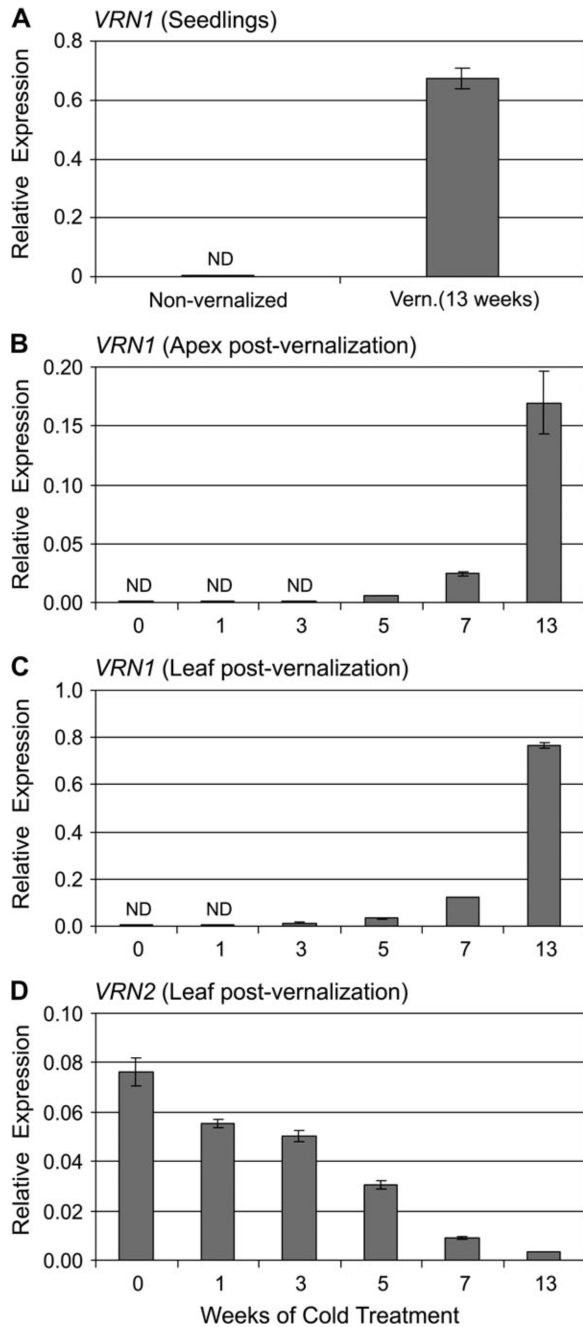

Fig. 7. The seed vernalization response of wheat. (A) Expression of *VRN1* in seeds/seedlings of the winter wheat Norstar before and after seed vernalization. (B) Expression of *VRN1* in the shoot apex at the third leaf stage, after seed vernalization treatments of different durations. (C) Expression of *VRN1* in leaves (second leaf) at the third leaf stage, in long days, after seed vernalization treatments of different durations. (D) Expression of *VRN2* in leaves (second leaf) at the third leaf stage, in long days, after seed vernalization treatments of different durations. *VRN1* and *VRN2* transcript levels were assayed by quantitative RT-PCR and are shown relative to *ACTIN*. ND denotes no expression detected.

of the expression patterns of flowering-time genes during and after seed vernalization supports the hypothesis that activation of *HvVRN1* mediates the low-temperature flowering response in temperate cereals.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Expression of *APETALA1*-like and *SEPALLATA*-like MADS box genes in seeds at normal or vernalizing temperatures.

Fig. S2. Flowering time of the winter wheat cv. Norstar after seed vernalization treatments, in long days.

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