Short Vegetative Phase-Like MADS-Box Genes Inhibit Floral Meristem Identity in Barley[1][W][OA]

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Analysis of the functions of *Short Vegetative Phase* (*SVP*)-like MADS-box genes in barley (*Hordeum vulgare*) indicated a role in determining meristem identity. Three *SVP*-like genes are expressed in vegetative tissues of barley: *Barley MADS1* (*BM1*), *BM10*, and *Vegetative to Reproductive Transition gene 2*. These genes are induced by cold but are repressed during floral development. Ectopic expression of *BM1* inhibited spike development and caused floral reversion in barley, with florets at the base of the spike replaced by tillers. Head emergence was delayed in plants that ectopically express *BM1*, primarily by delayed development after the floral transition, but expression levels of the barley *VRN1* gene (*HvVRN1*) were not affected. Ectopic expression of *BM10* inhibited spike development and caused partial floral reversion, where florets at the base of the spike were replaced by inflorescence-like structures, but did not affect heading date. Floral reversion occurred more frequently when *BM1* and *BM10* ectopic expression lines were grown in short-day conditions. *BM1* and *BM10* also inhibited floral development and caused floral reversion when expressed in Arabidopsis (*Arabidopsis thaliana*). We conclude that *SVP*-like genes function to suppress floral meristem identity in winter cereals.

During the life cycle of a plant, the shoot apical meristem progresses through three phases of development: vegetative, inflorescence, and floral (Poethig, 1990). In each phase, the apical meristem produces a different set of organs. The vegetative meristem produces leaves, the inflorescence meristem produces leaves and floral meristems to form the inflorescence, and the floral meristem produces the organs that form the flower. The different phases of meristem development are controlled by genes that establish and maintain meristem identity.

The shift from vegetative to inflorescence meristem identity, the floral transition, marks the beginning of the reproductive growth phase and is an important determinant of flowering time. In Arabidopsis (*Arabidopsis thaliana*), the *Short Vegetative Phase* (*SVP*) gene encodes a MADS-box transcription factor that delays the floral transition (Hartmann et al., 2000). Mutations that disrupt *SVP* cause early flowering (Hartmann et al., 2000), whereas ectopic expression of *SVP* results in late flowering. Ectopic expression of *SVP* also inhibits floral meristem identity, causing floral abnormalities such as the conversion of sepals and petals to leaf-like structures (Brill and Watson, 2004; Masiero et al., 2004) and causing inflorescence-like structures to develop within flowers (Brill and Watson, 2004). The development of inflorescences within flowers indicates that meristematic cells within the flower have lost floral identity and have formed an inflorescence instead of floral organs, a phenomenon known as floral reversion (Tooke et al., 2005). Presumably, ectopic expression of *SVP* causes floral reversion by interfering with a mechanism that maintains floral meristem identity.

The Arabidopsis gene, *AGAMOUS-LIKE 24* (*AGL24*), is closely related to *SVP* (Yu et al., 2002; Michaels et al., 2003). Unlike *SVP*, *AGL24* promotes the floral transition. Mutations that disrupt *AGL24* cause late flowering, whereas overexpression of *AGL24* accelerates flowering (Yu et al., 2002; Michaels et al., 2003). *AGL24* is expressed during vegetative development and is induced by treatments that accelerate floral transition, such as vernalization (prolonged exposure to low temperatures), long days, or the application of gibberellins (Yu et al., 2002; Michaels et al., 2003). These data suggest that *AGL24* acts to promote floral transition in response to vernalization and long-day conditions (Yu et al., 2002; Michaels et al., 2003). Although *AGL24* has the opposite effect on flowering time compared to *SVP*, plants that ectopically express *AGL24* exhibit floral abnormalities similar to those caused by ectopic expression of *SVP*, and ectopic expression of *AGL24* also causes floral reversion. Thus, *AGL24* promotes the floral transition but inhibits floral meristem identity. It has been suggested that *AGL24* promotes inflorescence meristem identity (Yu et al., 2004).

Cereals and related grass species follow a pattern of development similar to that of other plants. When

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floral transition occurs, the shoot apex elongates and floral primordia form, resulting in the appearance of distinctive double ridges. Then, as the shoot apex differentiates into a compact inflorescence, the floral primordia give rise to spikelets that produce the floral meristems that develop into florets (flowers). In winter cereals, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), the floral transition can be accelerated by long days or by prolonged cold treatment (vernalization) and is also subject to genetic variation that affects the number of leaves that develop prior to the floral transition (Boyd et al., 2003).

The induction of the floral transition by vernalization is mediated by the *VRN1* gene in winter cereals and related grass species such as *Lolium perenne* (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Andersen et al., 2006). *VRN1* encodes an *APETALA1* (*AP1*)-like MADS-box gene that is induced by exposure to long periods of cold and promotes the floral transition (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). It is not known how cold causes activation of *VRN1* expression during vernalization. One suggestion is that an *SVP*-like gene, *Vegetative to Reproductive Transition gene 2* (*VRT2*, or *HvVRT2* for the barley homolog), mediates induction of *VRN1* in vernalized plants (Kane et al., 2005). In wheat, *VRT2* is expressed in vegetative plants. During cold treatment, *VRT2* expression decreases gradually in contrast to the gradual increase in expression of *VRN1* (Kane et al., 2005). The expression patterns of *VRT2* and *VRN1* during cold treatment of wheat are consistent with the hypothesis that *VRT2* represses *VRN1* and that down-regulation of *VRT2* by cold allows expression of *VRN1*. There is, however, no direct evidence that *VRT2* regulates *VRN1*. In the vernalization-responsive grass *L. perenne*, overexpression of the *VRT2* homolog, *L. perenne MADS-Box gene 10* (*LpMADS10*), does not affect the timing of flowering (Petersen et al., 2006). Thus, it remains unclear whether *SVP*-like genes regulate flowering time in grasses.

We have investigated the potential roles of *SVP*-like genes in the vernalization response of barley, an important crop and a useful diploid model for winter cereal crops. We examined the phenotypes of transgenic plants with altered expression levels for two of the barley *SVP*-like genes and examined whether *SVP*-like genes are likely to regulate expression of the barley *VRN1* gene (*HvVRN1*). We show that in barley, *SVP*-like genes regulate meristem identity but are unlikely to mediate derepression of *HvVRN1* during vernalization.

RESULTS

Three *SVP*-Like Genes Are Expressed in Vegetative Tissues of Barley But Are Repressed during Spike Development

Three *SVP*-like genes were isolated from a barley cDNA library: *Barley MADS1* (*BM1*; Schmitz et al., 2000), *HvVRT2* (Kane et al., 2005), and a third gene, designated *BM10* (GenBank EF043040; Supplemental Fig. S1). Phylogenetic analysis shows that *SVP*-like genes of barley are more closely related to each other than to either *SVP* or *AGL24*, suggesting that *SVP*-like genes have diverged separately in monocots and dicots (Fig. 1). Among the barley *SVP*-like genes, *BM10* is more closely related to *HvVRT2* (77% amino acid sequence identity) than *BM1* (51% amino acid sequence identity). All three genes are expressed in a range of vegetative organs (Supplemental Fig. S2).

The relationship between the floral transition and expression of the *SVP*-like genes was examined in an early flowering spring barley variety (cv Golden Promise). Expression levels of *BM1*, *BM10*, and *HvVRT2* were monitored at weekly intervals in whole plants during the first 4 weeks of development. The floral transition, as indicated by the double ridge stage of apex development, occurred after approximately 18 d. *HvVRT2* and *BM1* were expressed at similar levels throughout the developmental time course (Fig. 2A). *BM10* expression decreased slightly after 21 d of growth (to 0.6-fold

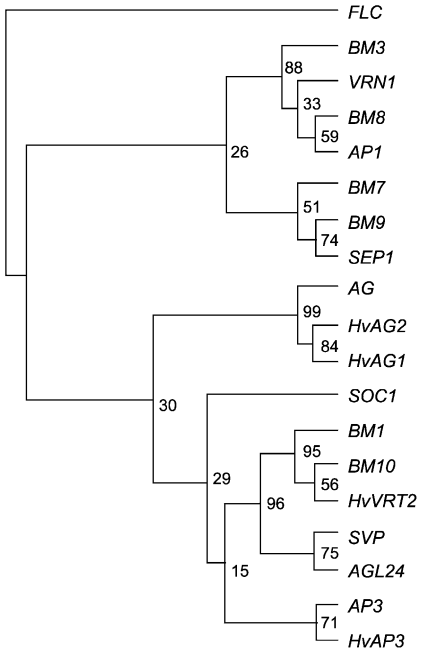

Figure 1. Phylogeny of barley MADS-box genes. A consensus phylogenetic tree created using the protein parsimony method of the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html). Bootstrap support values from 1,000 bootstrap replicates are shown for each node as percentages. Analysis was performed on an alignment of the amino acid sequences of the MADS-box domains (the region equivalent to amino acids 1–62 from alignment in Supplemental Fig. S1) of: *AGAMOUS* (*AG*, NP 567569), *AGL24* (NM118587), *SVP* (NM127820), *BM10* (EF043040), *HvVRT2* (DQ201168), *BM1* (AJ249142), *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*, NM130128), *SEPALLATA1* (*SEP1*, NM121585), *FLOWERING LOCUS C* (*FLC*, NM121052), *AP3* (NM115294), *AP1* (NM105581), *HvVRN1* (*VRN1*, AJ249144), *BM3* (AJ249143), *Hordeum vulgare AGAMOUS-like 1* (*HvAG1*, AF486648), *Hordeum vulgare AGAMOUS-like 2* (*HvAG2*, AF486649), *BM7* (AJ249145), *BM8* (AJ249146), *BM9* (AJ249147), and *Hordeum vulgare APETALA3* (*HvAP3*, AY541065).

of starting level). In comparison, expression of *HvVRN1* increased after 21 d (Fig. 2A).

Expression levels of *BM1*, *BM10*, and *HvVRT2* were then monitored in the developing shoot apex. Expression levels of all three genes were similar in vegetative apices (14 d, vegetative meristem) and in apices at the double ridge stage (18 d, inflorescence meristems) but were reduced at later stages of apical development (42 d, floral meristems; Fig. 2B). At this stage, floral organ differentiation was visible (Supplemental Fig. S3). Expression of *HvVRN1* increased around the time of floral transition (10-fold) and remained high throughout subsequent stages of apex development (Fig. 2B).

BM1, *BM10*, and *HvVRT2* Are Not Regulated by Day Length

Day length influences the timing of floral transition in cereals. Long days have been reported to repress *HvVRT2* expression during cold treatment (Kane et al., 2005). We examined the influence of day length on expression of *BM1*, *BM10*, and *HvVRT2* during long-day induction of the floral transition in a day-length responsive spring barley variety (cv Icheon Naked). Plants were grown in short days then shifted to inductive long-day conditions. Plants shifted to long days underwent floral transition, as indicated by the presence of double ridges at the shoot apex, within 2 weeks, whereas plants maintained in short days remained vegetative. Plants in the long-day treatment flowered 60 d earlier than plants maintained in short days (head emergence after 50 ± 1 d versus 111 ± 1 d). After 7 d, expression levels of *BM1*, *BM10*, and *HvVRT2* were similar in both treatments (Fig. 3A). In comparison, expression of *HvFT*, a key gene in the photoperiod response pathway (Turner et al., 2005), was strongly induced by the long-day treatment (Fig. 3B). Expression of *HvVRN1* was also induced by long days

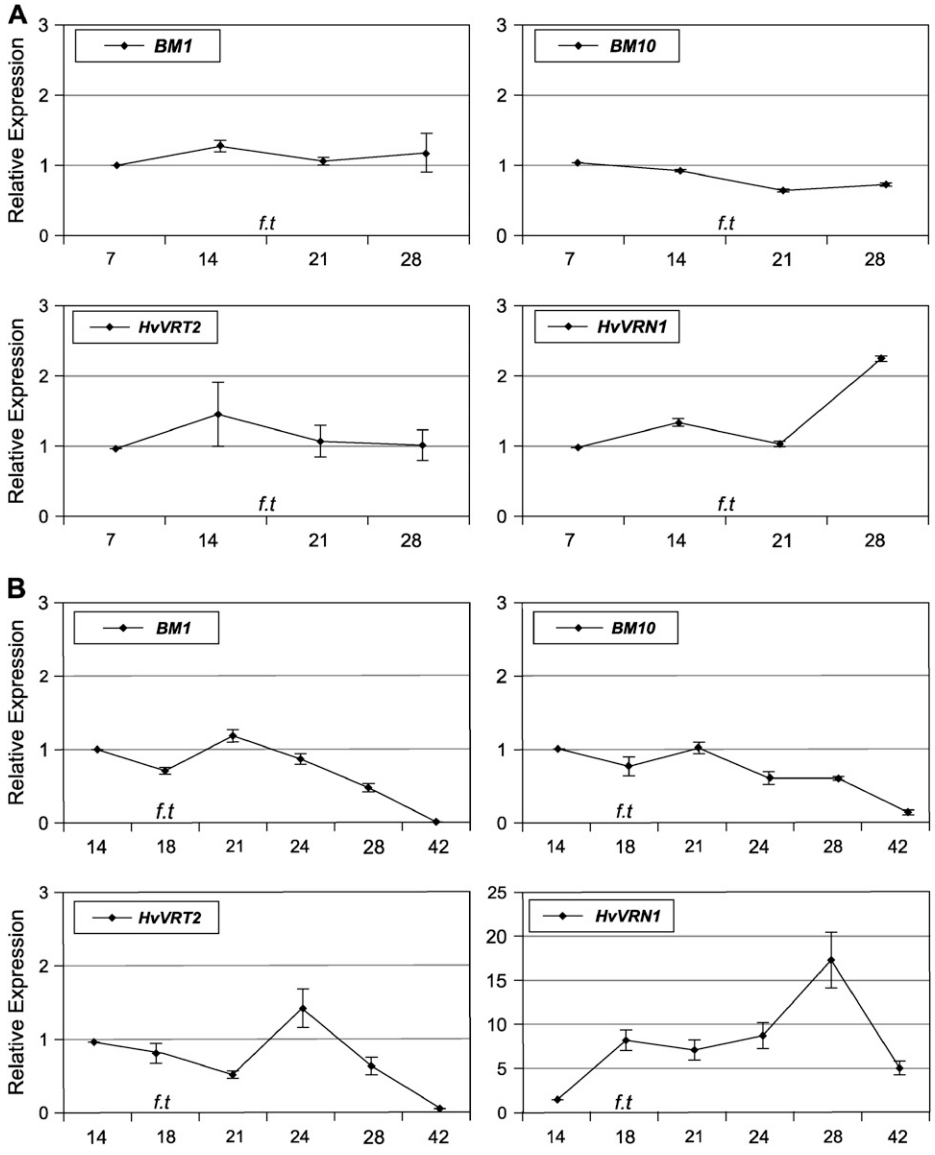

Figure 2. Developmental regulation of *BM1*, *BM10*, *HvVRT2*, and *HvVRN1*. A, Relative expression levels of *BM1*, *BM10*, *HvVRT2*, and *HvVRN1* assayed by qRT-PCR and normalized to *ACTIN* (see "Materials and Methods") in RNA from plants of different ages (cv Golden Promise, minus roots). Expression level is shown relative to the initial time point (7-d-old plants). B, Relative expression levels of *BM1*, *BM10*, *HvVRT2*, and *HvVRN1* in isolated shoot apices harvested from plants of different ages. Expression is shown relative to the initial time point (RNA from apices of 14-d-old plants). *f.t* denotes the time point of the floral transition, as indicated by the appearance of double ridges on the shoot apex. Error bars show SE.

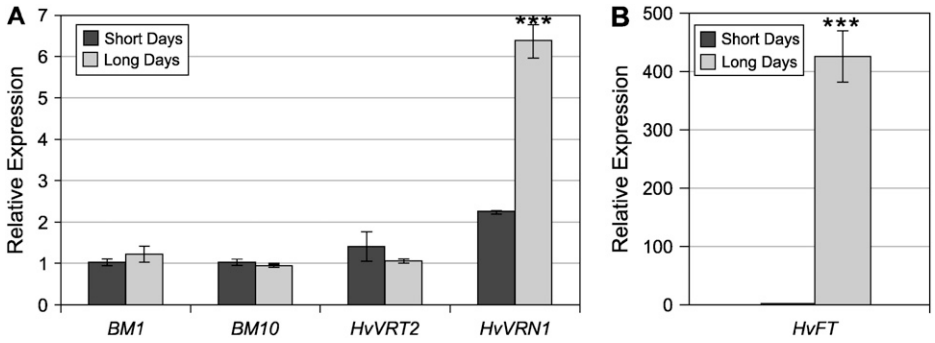

Figure 3. The effect of short or long day lengths on expression levels of *BM1*, *BM10*, and *HvVRT2*. A, Relative expression levels of *BM1*, *BM10*, *HvVRT2*, and *HvVRN1*, assayed by qRT-PCR, in RNA from plants (cv Icheon Naked, minus roots) that were maintained in short days (8 h dark/16 h light) or shifted to long days (16 h light/ 8 h dark) for 7 d. Expression is shown relative to the initial time point (14-d-old plants in short days). B, Expression levels of *HvFT*, normalized to *ACTIN*, in the same RNA samples. Error bars show SE. Asterisks indicate *P* values of Student's *t* tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 3A), as has been reported previously for this barley variety (Trevaskis et al., 2006). These data suggest that, in barley, transcriptional repression of *SVP*-like genes is not required to promote floral transition in response to long-day conditions.

BM1, *BM10*, and *HvVRT2* Are Induced by Cold

It has been reported that *VRT2* is repressed during cold treatment in wheat (Kane et al., 2005). We found that *BM1*, *BM10*, and *HvVRT2* were induced by cold in a vernalization-responsive winter barley (cv Sonja:

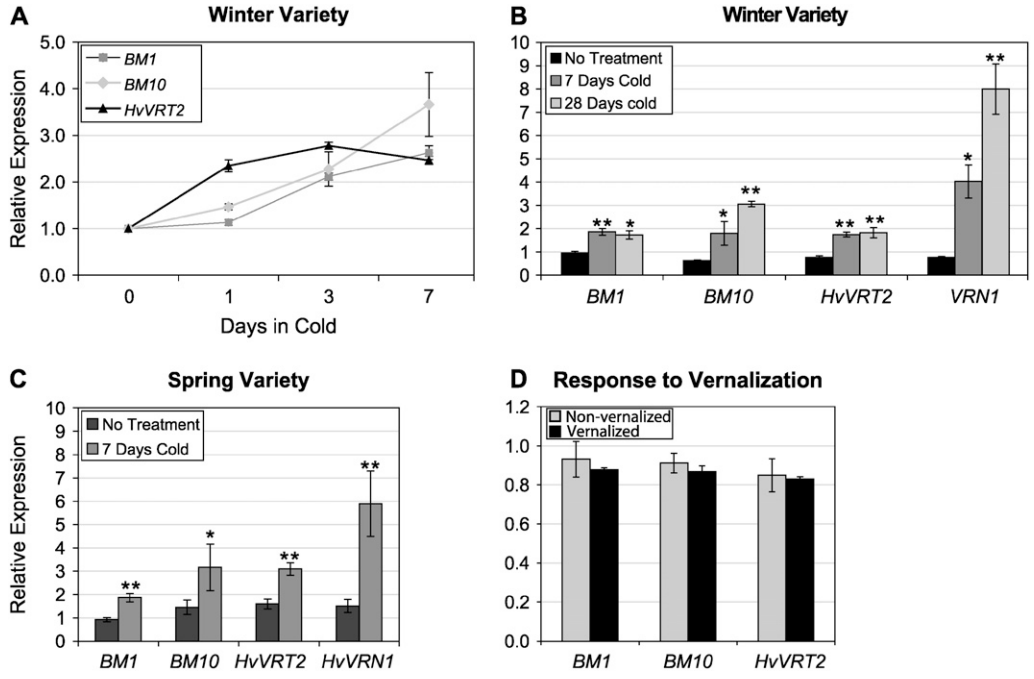

Figure 4. *BM1*, *BM10*, and *HvVRT2* are induced by cold. A, Relative expression levels of *BM1*, *BM10*, and *HvVRT2*, assayed by qRT-PCR, in a winter variety (cv Sonja, minus roots) after 0, 1, 3, or 7 d cold treatment (4°C). Expression is shown relative to the initial time point (14-d-old plants grown in standard glasshouse conditions). B, Relative expression levels of *BM1*, *BM10*, *HvVRT2*, and *HvVRN1*, assayed by qRT-PCR, in a winter barley variety (cv Sonja, minus roots) maintained in normal glasshouse conditions for 7 d (no treatment) or after 7 or 28 d of cold treatment. Expression is shown relative to the initial time point (14-d-old plants in standard glasshouse conditions). C, Expression levels of *BM1*, *BM10*, *HvVRT2*, and *HvVRN1*, assayed by qRT-PCR, in plants of a spring barley cultivar (cv Golden Promise, minus roots) that were maintained in normal glasshouse conditions (no treatment) or shifted to cold conditions (4°C) for 7 d. Expression is shown relative to the initial time point (14-d-old plants in normal glasshouse temperatures). Error bars show SE. D, Expression levels of *BM1*, *BM10*, and *HvVRT2* assayed by qRT-PCR in plants (cv Sonja, minus roots) that were imbibed and vernalized for 9 weeks then reacclimatized to standard glasshouse conditions for a week. Expression is shown relative to control nonvernalized plants at the same developmental stage (two leaves, vegetative shoot apex). Differences were nonsignificant by Student's *t* test. Error bars show SE. Asterisks indicate *P* values of Student's *t* tests: *, $P < 0.05$; **, $P < 0.01$.

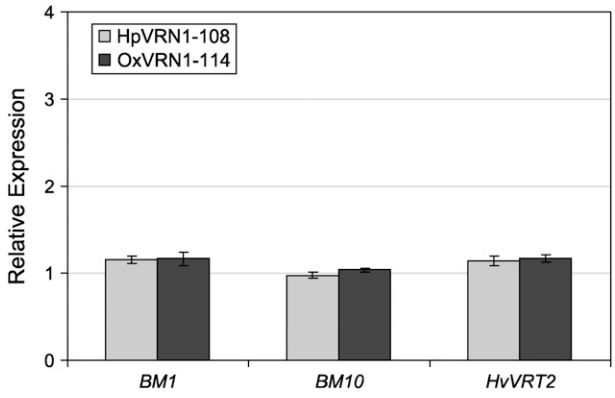

Figure 5. *HvVRN1* does not regulate transcription of *BM1*, *BM10*, or*HvVRT2*. Expression levels of *BM1*, *BM10*, and *HvVRT2*, assayed by qRT-PCR, in RNA from transgenic plants that carry either a RNAi construct against *HvVRN1* (line HpVRN1-108) or a *VRN1* overexpression construct (line OxVRN1-114). Expression is shown relative to a wild-type plant (14-d-old plants minus roots). Similar results were obtained in a second independently transformed line for each construct. Error bars show SE. Differences were nonsignificant by Student's *t* test.

Hvvrn1, *HvVRN2*). Cold induction of *BM1* and *HvVRT2* occurred within 3 d (Fig. 4A; Supplemental Fig. S4A), but longer cold treatments did not cause further increases in expression (Fig. 4B). Expression levels of *BM10* also increased within 3 d of cold treatment and increased further with longer cold treatments (Fig. 4, A and B). Cold treatment also induced *HvVRN1* expression, as has been reported previously for winter barley varieties (Trevaskis et al., 2003). In a spring barley, (cv Golden Promise: *HvVRN1*, *Hvvrn2*), cold treatment also induced *BM1*, *BM10*, and *HvVRT2*. This correlated with induction of *HvVRN1* (Fig. 4C; Supplemental Fig. S4B). These data are not consistent with *HvVRT2* being a repressor of *HvVRN1*.

The effect of vernalization on *BM1*, *BM10*, and *HvVRT2* expression levels was examined in a vernalization-responsive barley winter variety (cv Sonja). *BM1*, *BM10*, or *HvVRT2* expression levels did not differ between vernalized and nonvernalized plants (Fig. 4D). Expression of *HvVRN1* was strongly induced by the same treatment (250 ± 40.3) and heading date was accelerated (Trevaskis et al., 2003). Thus, it seems unlikely that repression of *HvVRT2* is required for induction of *HvVRN1* or the acceleration of floral transition by vernalization.

VRN1 Does Not Regulate *SVP*-Like Genes in Winter Cereals

Cold treatment of plants induced expression of both *HvVRN1* and *SVP*-like genes. We examined whether *HvVRN1* might regulate expression of *SVP*-like genes. Expression levels of *BM1*, *BM10*, and *HvVRT2* were compared between transgenic plants (cv Golden Promise) that overexpress *VRN1* or have reduced levels of *HvVRN1* transcript. Reverse transcription (RT)-PCR quantification showed that these lines had *HvVRN1* expression levels that were 21- ± 0.25-fold or 0.30- ± 0.01-fold that of wild-type plants, respectively, a difference that is clearly visible by RNA gel-blot analysis (Supplemental Fig. S5A). These changes did not affect expression of *BM1*, *BM10*, or *HvVRT2* (Fig. 5). Similarly, in wheat, *VRT2* expression did not vary between spring and winter near-isogenic lines of the Triple Dirk series (Pugsley, 1971), which express *VRN1* to different levels (Trevaskis et al., 2003; Supplemental Fig. S5B). It is unlikely that *VRN1* directly regulates expression of *VRT2*.

BM1 or *BM10* Does Not Regulate Expression of *HvVRN1*

To investigate the functions of *SVP*-like genes in barley, we altered the expression levels of *BM1* and *BM10* (*HvVRT2-like*) in transgenic barley. These genes were chosen because they are phylogenetically distinct (Fig. 1) and have different expression profiles during cold treatment. Initially, the effects of reducing the levels of *BM1* or *BM10* expression levels were investigated using gene-specific RNA interference (RNAi) constructs. More than 50 independent transgenic lines were produced for each construct. Some lines showed reduced expression levels for the targeted genes (Supplemental Fig. S6), but none of the lines generated for either construct showed any phenotypic abnormalities or any change in heading date (Supplemental Fig. S7).

As reduced expression of *BM1* or *BM10* did not elicit any abnormal phenotypes, *BM1* or *BM10* were expressed under the control of the constitutive maize (*Zea mays*) *UBIQUITIN* promoter (Supplemental Fig. S8). The *BM1* overexpression construct (*OxBM1*) delayed head emergence by approximately 10 d, whereas the *BM10* overexpression construct (*OxBM10*) did not influence heading date (Supplemental Fig. S7). Comparison of apex lengths, a measure of floral development, between plants that overexpress *BM1* and control siblings showed that overexpression of *BM1* delayed the floral transition by approximately 3 d (Fig. 6, A and B) and that the delay in head emergence in *BM1* overexpression lines (approximately 10 d) must be due primarily to delayed floral development after the floral transition. *HvVRN1* expression levels were similar in plants that ectopically express *BM1* or *BM10* and sibling null control lines (Fig. 6C), and *HvVRN1* expression could be induced by cold treatment in plants that ectopically express *BM1* or *BM10* (Fig. 6D). Thus, the delay of heading date caused by *OxBM1* is not due to transcriptional repression of *HvVRN1*.

Ectopic Expression of *BM1* or *BM10* Inhibits Floral Development and Causes Floral Reversion

Ectopic expression of *BM1* caused a number of phenotypes in the spike where *BM1* is not normally expressed. The rachis internodes were elongated giving the spike a concertina phenotype (Fig. 7, A and B) and the vestigial florets normally found on two-row cultivars were reduced in size (Fig. 7B). In the fertile florets, the palea and lemma were elongated and more

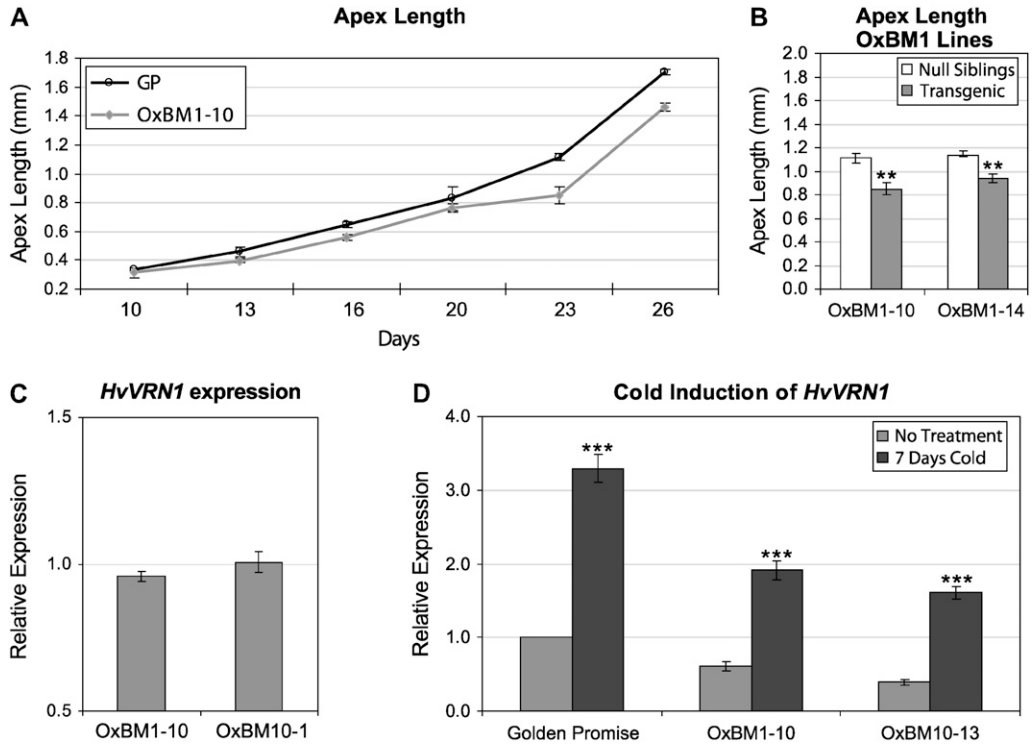

Figure 6. Ectopic expression of *BM1* delays the floral transition, but *HvVRN1* levels are not altered. A, Apex length (average length in millimeters of apices from five plants) of wild-type plants, cv Golden Promise (GP), or transgenic plants homozygous for *OxBM1* (line OxBM1-10) harvested at different time points. B, Comparison of the average length of the shoot apex after 23 d for homozygous transgenic and sibling null control plants from two independent *BM1* overexpression lines. C, *HvVRN1* expression levels in *BM1* (line 10) or *BM10* (line 1) overexpression lines (14-d-old plants minus roots). Expression levels were assayed by qRT-PCR and are shown relative to the level of expression in control sibling null plants. D, *HvVRN1* expression, as assayed by qRT-PCR, in plants (whole plants minus roots) homozygous for the *BM1* (*OxBM1-10*) or *BM10* (line 13) overexpression construct that were grown for 14 d then either moved to cold conditions for 7 d or maintained in standard glasshouse conditions (no treatment). Expression is shown relative to the level of expression in Golden Promise plants from the control treatment. Error bars show SE. Asterisks indicate *P* values of Student's *t* tests: **, $P < 0.01$; ***, $P < 0.001$. Similar results were obtained in a second independently transformed line for each construct.

leaf like (Fig. 7C), whereas the lodicules were smaller than in wild-type plants (Fig. 7D). These phenotypes are all indicative of a loss of floral characteristics and a shift toward stem characteristics; unlike the spike, the internodes of cereal stems are elongated and the nodes bear leaves but not flowers.

In a small number of T0 lines (2/50 for *BM1* lines), tillers developed at the base of spikes at positions where florets would normally be located (Fig. 8, A–C). This suggests that ectopic expression of *BM1* converts a floral meristem to a vegetative meristem to give rise to tillers from within the spike. This effect is analogous to floral reversion in Arabidopsis. Floral reversion occurred at a higher frequency when plants were grown in short days. For example, when 10 plants overexpressing *BM1* (line 10) were grown in short days, four failed to produce heads and six produced heads that showed profuse ectopic tillering, with multiple tillers arising from within the primary spike (Fig. 8C). No ectopic tillering was observed when the same line was grown in long days or in a control sibling null grown in short days. Spike development was severely disrupted when ectopic tillering occurred. The apex progressed past floral transition, but often florets did not develop fully (Fig. 8, B and C) and plants were infertile when grown in short days, even when florets did develop.

Overexpression of *BM10* caused very similar spike and floret phenotypes in long-day-grown plants (Fig. 7A). Moreover, the florets at the base of the spike frequently failed to develop in *BM10* overexpression lines and reverted to inflorescence-like structures (Fig. 8, D and E), suggesting that expression of *BM10* causes the floral meristem to revert to an inflorescence, a partial floral reversion. As was the case for *BM1* overexpression lines, overexpression of *BM10* also caused complete floral reversion in short days, giving rise to ectopic tillers from within the spike. The same phenotypes did not occur in null sibling controls.

Expression of Barley *SVP*-Like Genes in Arabidopsis Causes Floral Reversion Phenotypes

When *BM1* and *BM10* were expressed in Arabidopsis (C24 ecotype) under the control of the cauliflower

Figure 7. Abnormal spike morphology in plants transformed with OxBM1 or OxBM10 constructs. Spike phenotypes caused by ectopic expression of *BM1* or *BM10*. A, A spike from a wild-type plant (cv Golden Promise, left) compared to spikes from a transgenic plant homozygous for *OxBM1* (line OxBM1-10, middle) or OxBM10 (line OxBM10-13, right). Spikes were taken at head emergence. B, Comparison of dissected inflorescence stems from a wild-type plant (left) and a plant overexpressing *BM1* (OxBM1-10, right) showing the elongation of rachis internodes (ri) and the reduced size of the vestigial florets (vf). C, A comparison of florets from a wild-type plant (left) and a plant overexpressing *BM1* (OxBM1-10, right) showing the elongation of the lemma (l). D, As above, but the lemma has been removed to reveal the elongation of the palea (p) and the reduction in size of the lodicules (ld).

mosaic virus 35S promoter (*35S*), floral abnormalities similar to those caused by ectopic expression of *SVP* resulted. These included conversion of petals and sepals to leaf-like structures, enlargement of the gynoecium, and production of stipitate ovaries, where the base of the ovary elongates (Fig. 9, A–C). The presence of leaves and elongation of internodes are features of the inflorescence and are indicative of a shift toward inflorescence identity in the flower. The flowers of Arabidopsis plants expressing *BM1* or *BM10* also underwent frequent floral reversion events, where an inflorescence formed within the flower (Fig. 9, D–F). This occurred in two different locations within flowers: at the end of the gynoecium or more commonly within the axil of the sepal. Sometimes this occurred more than once from a single flower (Fig. 9, D and E).

DISCUSSION

We have investigated the relationship between the timing of the floral transition and expression levels of three *SVP*-like genes in barley. We found no evidence that transcriptional repression of these *SVP*-like genes is required for floral transition to occur. All three genes are expressed at similar levels in vegetative and inflorescence meristems. Moreover, we found no evidence that transcriptional repression of *BM1*, *BM10*, or *HvVRT2* is required to accelerate the floral transition in response to vernalization or long days. Expression of all three genes did decrease at later stages of apex development, suggesting that reduced expression levels of *BM1*, *BM10*, and *HvVRT2* may be required for floral development.

Ectopic expression of *BM1* or *BM10* caused a shift toward vegetative characteristics in the spikes of barley, consistent with the hypothesis that down-regulation of *SVP*-like genes is important during floral development. Ectopic expression of *Oryza sativa MADS gene 22* and *LpMADS10* cause similar inhibition of floral characteristics when ectopically expressed in rice (*Oryza sativa*) or *L. perenne*, respectively, but neither gene affects heading date (Sentoku, personal communication; Petersen et al., 2006). It has been suggested that these genes must regulate meristem identity (Sentoku et al., 2005; Petersen et al., 2006).

In long days, ectopic expression of *BM1* caused floral primordia to revert to vegetative meristems in some lines, resulting in the appearance of ectopic tillers within the spike. Similarly, ectopic expression of *BM10*

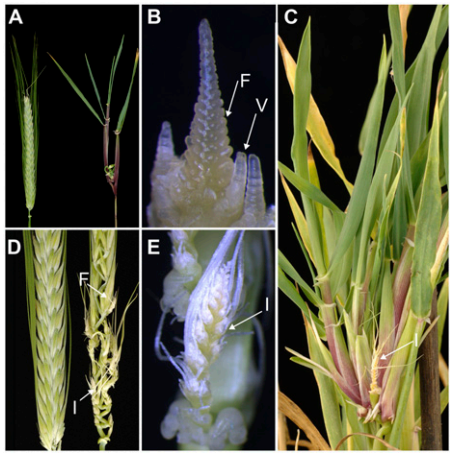

Figure 8. Floral reversion in plants transformed with *OxBM1* or *OxBM10* constructs. A, A spike from a short-day-grown wild-type plant (left) compared to a spike from a plant overexpressing *BM1* (OxBM1-10, right) grown in the same conditions. B, The shoot apex (250× magnification) from a short-day-grown plant overexpressing *BM1* (OxBM1-10) showing the conversion of floral primordia (F) to vegetative meristems (V). C, The severe floral reversion phenotype caused by ectopic expression of *BM1* in short days (OxBM1-10). Multiple tillers can be seen radiating from the base of the inflorescence (I) that has not developed fully. D, Comparison of a wild-type spike (left) and a spike from a *BM10* overexpression line (OxBM10-13, right) showing conversion of florets (F) to inflorescences (I). E, A magnified view (90× magnification) of inflorescences (I) that have developed in the place of florets in the same line.

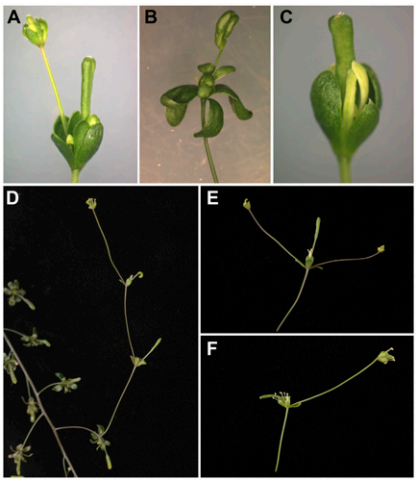

Figure 9. Floral reversion and abnormal floral phenotypes in Arabidopsis plants that express *BM1* or *BM10*. A, Flower from an Arabidopsis plant transformed with the *35S*::*BM1* construct showing the conversion of sepals and petals to leaf-like organs and floral reversion, where an inflorescence has arisen from within the flower. B, The flower from an Arabidopsis plant transformed with the *35S*::*BM10* construct showing the conversion of sepals and petals to leaf-like organs. C, Stipitate ovary and enlargement of the gynoecium in a flower from an Arabidopsis plant expressing *BM10*. D, Multiple floral reversion events in a plant expressing *BM1* plant. E and F, Floral reversion events in plants expressing *BM10*.

caused floral meristems to revert to inflorescence-like structures. Thus, both genes disrupt floral meristem identity and cause floral reversion when expressed ectopically in barley. For both genes, the effect of ectopic expression was more extreme in short days and often completely blocked normal floral development after floral transition. These floral reversion phenotypes strongly support the hypothesis that *SVP*-like genes regulate meristem identity in cereals and related grass species. The slight delay of the floral transition caused by ectopic expression of *BM1* shows that this gene may also inhibit inflorescence meristem identity in long days and could influence the timing of the floral transition in some conditions or in some genetic backgrounds. The absence of abnormal phenotypes in barley plants with reduced expression of *BM1* or *BM10* suggests there is functional redundancy between *SVP*-like genes in barley, although it is also possible that none of the lines generated (over 50 for each construct) had sufficiently reduced expression of the target gene to reveal any phenotypes.

Expression of *BM1* or *BM10* caused floral reversion when expressed in Arabidopsis (a dicot) and barley (a monocot). Expression of other *SVP*-like genes (e.g. *SVP* and *AGL24*) is known to cause floral reversion in Arabidopsis. The ability of *SVP*-like genes to trigger floral reversion is not an artifact of ectopic expression; in tomato (*Lycopersicon esculentum*), indeterminant sympodial growth is achieved through successive floral reversions, where an inflorescence develops below the flower on an otherwise determinant floral branch (Szymkowiak and Irish, 2006). A *SVP*-like gene, *JOINTLESS*, is required to generate an inflorescence meristem (the sympodial meristem) below the flower and is essential for sympodial growth (Szymkowiak and Irish, 2006). Floral reversion is also caused by mutations that inhibit floral meristem identity, such as *ap1* (for review, see Tooke et al., 2005). In the *ap1* mutant, floral reversion occurs more frequently in short-day conditions (Okamuro et al., 1997). In barley, we found that short-day conditions increased the frequency and severity of floral reversion in plants that ectopically express *BM1* or *BM10*. This suggests that day length conditions influence floral meristem identity in Arabidopsis and barley.

SVP-like genes may be components of a conserved regulatory mechanism that controls meristem identity in both dicots and monocots. This may involve interactions between SVP-like and AP1-like MADS-box proteins. In Arabidopsis, SVP and AGL24 interact with AP1 and members of a LEUNIG-SEUSS corepressor

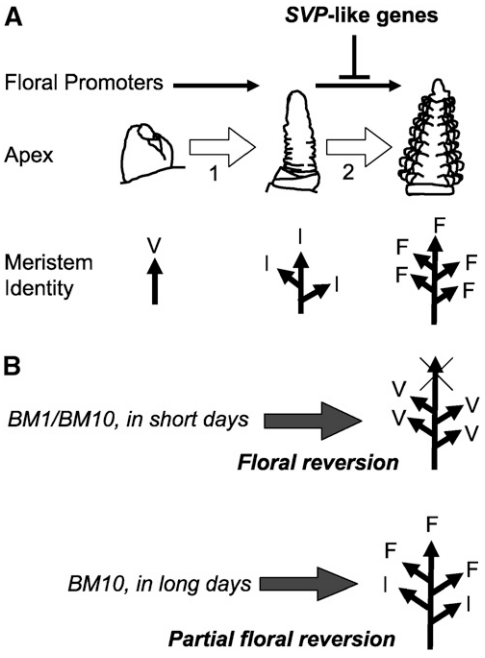

Figure 10. Schematic representation of the regulation of meristem identity in winter cereals by *SVP*-like genes. A, The shoot apex normally proceeds through two phase transitions: from vegetative to inflorescence, and from inflorescence to floral. Long days and vernalization accelerate these phase transitions through the activity of floral promoters such as *FT*, *VRN1*, or gibberellins. *SVP*-like genes are expressed in the vegetative and inflorescence apex to inhibit floral meristem identity and prevent precocious development of floral organs, particularly under short days and in cold conditions. B, Ectopic expression of *BM1* or *BM10* inhibits floral meristem identity and retards floral development. In short days, where floral promoters are less active, ectopic expression of *BM1* or *BM10* has a stronger effect and causes the floral meristems to revert to vegetative meristems. In long days, where floral promoters promote floral development this is sufficient to weaken floral characteristics and cause occasional floral reversion (*BM1*) or partial floral reversion (*BM10*).

complex to repress *AGAMOUS* during floral development (Gregis et al., 2006). Similar interactions between SVP-like and AP1-like proteins may be important for floral development in cereals and other grasses. In yeast (*Saccharomyces cerevisiae*) two-hybrid assays, LpMADS10 interacts with LpVRN1 (*LpMADS1*; Ciannamea et al., 2006; Petersen et al., 2006), and TaVRT2 interacts with VRN1 (Kane et al., 2005).

If *SVP*-like genes inhibit floral meristem identity in cereals, what role does this play during normal plant development? *BM1* and *BM10* may be expressed in vegetative and inflorescence tissues to prevent premature transition from inflorescence to floral meristem identity (Fig. 10). It seems likely that these *SVP*-like genes would delay the formation of floral meristems more during winter when temperatures drop and days are short, inducing the expression of *BM1* and *BM10* and enhancing the inhibition of floral meristem identity by these genes. These genes may therefore play an important role to repress floral development during winter, possibly to counteract induction of flowering by vernalization. According to this hypothesis, *BM1* and *BM10* act antagonistically to genes that promote floral meristem identity and developmental down-regulation of *BM1* and *BM10* some time after floral transition is required to allow floral development to proceed. *HvVRT2*, which is similar in sequence to *BM10* and has a similar expression pattern to *BM1* and *BM10*, may have a similar function.

Kane et al. (2005) reported that in short-day conditions, *HvVRT2* expression decreased after 77 d of cold treatment. This may have been caused by prolonged cold treatment (vernalization) or by the floral transition, which occurred at 70 d. We found that cold induced *HvVRT2* expression in vegetative barley plants and that this correlated with induction of *HvVRN1*. This is not consistent with the suggestion that derepression of *HvVRT2* is required for cold induction of *HvVRN1*. Another mechanism proposed to account for induction of *HvVRN1* by cold involves the *HvVRN2* floral repressor locus (Yan et al., 2004; Fu et al., 2005). We found that *HvVRN1* is induced by cold in the spring barley Golden Promise, which lacks *HvVRN2*, suggesting that cold induction of *HvVRN1* can occur independently of *HvVRN2*. Golden Promise also lacks the regulatory region in the first intron of *HvVRN1*, which is required for repression in non-vernalized plants (Fu et al., 2005), suggesting that this region is not critical for cold induction of *HvVRN1*. Identification of the regulatory components that mediate cold induction of *VRN1* will be an important step in understanding the vernalization response in winter cereals.

In summary, the phenotypes of barley and Arabidopsis plants that ectopically express *BM1* or *BM10* are consistent in showing inhibition of floral organ development and reversion of the floral meristems. Thus, the primary function of *SVP*-like genes in cereals seems to be in determining meristem identity. This is likely to be a conserved function of *SVP*-like genes per se.

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). Glasshouses had an average temperature of 19°C. For vernalization treatment, plants were imbibed in soil and placed at 4°C (12 h light/12 h dark) for 9 weeks, then reacclimatized to normal glasshouse temperatures for 1 week. Nonvernalized control plants were grown for 2 weeks at normal glasshouse temperatures to reach a developmental stage equivalent to the vernalized plants at the time of harvest. Plants were harvested at the middle of the light period for each treatment. For cold treatment, plants were harvested directly from cold growth chamber (4°C, 12 h light/12 h dark).

Library Screening

cDNA was synthesized from RNA isolated from vegetative tissues of a winter barley (*Hordeum vulgare* cv Igri). cDNA was packaged into the Stratagene λ-ZAP phagemid vector (Sydney) and screened at high or low hybridization stringency with probes from the MADS-box domain of the *FLOWERING LOCUS C* (*FLC*) gene of Arabidopsis (*Arabidopsis thaliana*; GenBank NM121052) according to established methods (Ausubel et al., 1994). Sequence alignments were generated using the ClustalW program (Chenna et al., 2003).

RNA Extraction and Gel-Blot Analysis

Total RNA was extracted using the method of Chang et al. (1993). Twenty micrograms of RNA was separated in formaldehyde gels and blotted to Hybond-N membrane (Amersham Biosciences) as described in Ausubel et al. (1994). RNA blots were hybridized with riboprobes and washed using the protocol of Dolferus et al. (1994). Riboprobes were synthesized using the Promega T7 transcription kit. Blots were visualized in a phosphoimager for varying lengths of time, dependant on signal intensity. The following primers were used to amplify gene-specific riboprobe fragments: *BM1*, 5′-ACTGCAGGCGTCCAAGATG-3′ and 5′-CCACACTATGCTATATATTCCC-3′; *BM10*, 5′-GGAAGTAACAGTAGTTGTC-3′ and 5′-ATACCAACAAGGTACATA-3′; *HvVRT2*, 5′-TCATCTGACTCTGTGATGACGG-3′ and 5′-AAGACAGTTACTGAATACCCAGGC-3′; *HvVRN1*, 5′-GGTGAGCCACATCAACGGCTGA-3′ and 5′-TTACATGGTAGATTACTCGTACAGCC-3′. In each case, the T7 polymerase promoter was incorporated into the reverse primer sequence.

RT-PCR and Real-Time PCR Analysis

Quantitative RT-PCR (qRT-PCR) was performed on a Rotor-Gene 2000 Real-Time Cycler (Corbett Research). Products were sequenced to ensure that products were gene specific. The *ACTIN* gene was used as a housekeeping control to correct for uneven amounts of sample and control cDNA templates. Relative expression levels of genes of interest were compared between cDNA samples using the Comparative Quantification analysis method (Rotogene-5 software, Corbett Research). This method uses information about the start of the exponential phase of amplification (take-off point) and the average amplification efficiency of each primer set to enable direct concentration comparison between different samples generating a relative concentration. 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. In all cases, primers specifically amplify cDNA and do not amplify genomic DNA products. Three technical repeats were performed on each cDNA sample. Two biological repeats were assayed, giving similar trends. Data from one biological repeat is presented. The following primers were used: *BM1*, 5′-AGAGGAGAACGCAAGGCTAAAGG-3′ and 5′-AGTTGAAGAGTGATAATCCGAGCCTGAG-3′; *BM10*, 5′-GCTCATCGTCTTCTCCTCCAC-3′ and 5′-CTCCTCGCCTCTCATCTGTC-3′; *HvVRT2*, 5′-AAGCTCTCCCAGTTCGCCAGCTCC-3′ and 5′-TTAGTCCGTCAAGTTCCTCACC-3′; *HvVRN1*, 5′-GCATAAGTTGGTTCTTCCTGGCTCTG-3′ and 5′-GCCTCATCATCTTCTCCACCAA-3′; *HvFT*, 5′-GCGACCCCAACCTTAGAGAG-3′ and 5′-CTCGGCAAAGTCCCTGGTG-3′. *ACTIN* primers have been described previously (Trevaskis et al., 2006).

Plant Transformation

For Arabidopsis transformation, overexpression constructs were made by fusing cDNA clones of *BM1* and *BM10* to the cauliflower mosaic virus 35S promoter, and the resulting overexpression cassettes were ligated into the pART27 binary vector (Gleave, 1992). Arabidopsis plants were transformed with the floral dip method (Clough and Bent, 1998). T1 and T2 generations were screened by PCR using gene-specific primers (same primer sets as for qRT-PCR). For barley transformation, overexpression constructs were made by fusing cDNA clones of *BM1*, *BM10*, or *VRN1* (GenBank BE431095) to the maize (*Zea mays*) ubiquitin promoter (Christensen et al., 1992), and the resulting overexpression cassettes were ligated into the pWBVEC8 binary vector (Wang et al., 1998). RNAi constructs were made by amplifying fragments designed to specifically silence *BM1* (5′-AGGGGAGGAGAGGATGGC-3′ and 5′-GCTTCGGGTAATTTCCAGCC-3′), *BM10* (5′-GGCAAGCTAGTGGTTGTTGATACCG-3′and 5′-ACGGGTCCTAGCAAATGCCAAACAG-3′), or *HvVRN1* (5′-TGGATCCGGTACCGCAACAAGATCAGACTCAGCC-3′ and 5′-TCTAGATATCAAATTCACATAAACAACATCC-3′), which were inserted into the pSTARLING vector (http://www.pi.csiro.au/rnai/vectors.htm). The resulting hairpin cassettes were then ligated into the pWBVEC8 binary backbone. Barley plants were transformed using Agrobacterium transformation of excised embryos (Tingay et al., 1997; Mathews et al., 2001). An average of 50 independent transformants was produced for each construct. T1, T2, and T3 plants were screened for segregation of transgenes using primers that amplify from the hygromycin selectable marker gene (5′-AAAAGCCTGAACTCACCGC-3′ and 5′-TCGTCCATCACAGTTTGCC-3′). Expression analysis was carried out on plants homozygous for the transgene and sibling null control lines, which did not inherit the transgene. For each construct, expression analysis was carried out on at least two independently transformed lines that had phenotypes typical of the majority of T1 transformants.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EF043040.

Supplemental Data

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

Supplemental Figure S1. An alignment of amino acid sequences encoded by *SVP*-like genes from barley and Arabidopsis.

Supplemental Figure S2. *BM1*, *BM10*, *HvVRT2*, and *HvVRN1* transcript levels in different organs of barley.

Supplemental Figure S3. Apex development in the spring variety Golden Promise.

Supplemental Figure S4. Cold induction of *HvVRT2* and *HvVRN1*.

Supplemental Figure S5. *HvVRN1* expression levels do not influence expression levels of *BM1*, *BM10*, or *HvVRT2*.

Supplemental Figure S6. Effective silencing of *BM1* and *BM10* by RNAi constructs.

Supplemental Figure S7. Days to head emergence in transgenic barley plants.

Supplemental Figure S8. Ectopic expression of *BM1* or *BM10*.

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