# Evolution of *VRN2*/*Ghd7*-Like Genes in Vernalization-Mediated Repression of Grass Flowering[1][OPEN]

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Flowering of many plant species is coordinated with seasonal environmental cues such as temperature and photoperiod. Vernalization provides competence to flower after prolonged cold exposure, and a vernalization requirement prevents flowering from occurring prior to winter. In winter wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), three genes *VRN1*, *VRN2*, and *FT* form a regulatory loop that regulates the initiation of flowering. Prior to cold exposure, VRN2 represses *FT*. During cold, *VRN1* expression increases, resulting in the repression of *VRN2*, which in turn allows activation of *FT* during long days to induce flowering. Here, we test whether the circuitry of this regulatory loop is conserved across Pooideae, consistent with their niche transition from the tropics to the temperate zone. Our phylogenetic analyses of *VRN2*-like genes reveal a duplication event occurred before the diversification of the grasses that gave rise to a *CO9* and *VRN2*/*Ghd7* clade and support orthology between wheat/barley *VRN2* and rice (*Oryza sativa*) *Ghd7*. Our *Brachypodium distachyon VRN1* and *VRN2* knockdown and overexpression experiments demonstrate functional conservation of grass *VRN1* and *VRN2* in the promotion and repression of flowering, respectively. However, expression analyses in a range of pooids demonstrate that the cold repression of *VRN2* is unique to core Pooideae such as wheat and barley. Furthermore, *VRN1* knockdown in *B. distachyon* demonstrates that the *VRN1*-mediated suppression of *VRN2* is not conserved. Thus, the *VRN1*-*VRN2* feature of the regulatory loop appears to have evolved late in the diversification of temperate grasses.

The initiation of flowering is a major developmental transition in the plant life cycle. When flowering initiates, shoot apical meristems shift from forming vegetative organs such as leaves to forming flowers. In many plant species, flowering occurs at a particular time of year in response to the sensing of seasonal cues such as changes in day length and temperature. In some plants adapted to temperate climates, exposure to the prolonged cold of winter (vernalization) results in the ability to flower in the next growing season (Chouard, 1960; Amasino, 2010). Although vernalization ultimately enables flowering, vernalization responsiveness is typically an adaptation to ensure that flowering does not occur prematurely in the fall season. This has obvious adaptive value; for example, many vernalization-responsive plants become established in the fall season (during which flowering would not lead to successful reproduction) and then rapidly flower in the spring when conditions for reproduction and seed maturation are optimal.

The grass family (Poaceae) originated approximately 70 million years ago as part of the tropical forest understory. However, grasses have since diversified across the globe occupying a variety of ecological niches (Kellogg, 2001). Exemplifying this, the ~3,800 species of grass subfamily Pooideae, including the economically important cereals wheat (*Triticum aestivum*, Triticeae), barley (*Hordeum vulgare*, Triticeae), oat (*Avena sativa*, Poeae), and rye (*Lolium perenne*, Poeae), have adapted to cool climates of both northern and southern hemispheres (Hartley, 1973; Grass Phylogeny Working Group, 2001; Edwards and Smith, 2010). Phylogenetic analyses indicate the above-mentioned species within Triticeae and Poeae are a closely related group often referred to as crown or core

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pooid grasses (Schneider et al., 2009; Grass Phylogeny Working Group II, 2012). The remaining noncore pooids are in tribes consecutively sister to the core pooids, including Brachypodieae that contains the emerging plant model *Brachypodium distachyon* (Meliceae and Stipeae; Brkljacic et al., 2011; Grass Phylogeny Working Group II, 2012; Fig. 1).

It is hypothesized that vernalization responsiveness evolved early during the diversification of Pooideae, as a key adaptation allowing for their transition into the temperate zone (Preston and Sandve, 2013; Fjellheim et al., 2014). Within core Pooideae, many species have been characterized as vernalization responsive (Heide, 1994; Grass Phylogeny Working Group, 2001; Grass Phylogeny Working Group II, 2012). However, it is unclear how widespread vernalization responsiveness is outside core pooids and whether pooids with this trait share a conserved ancestral vernalization pathway. To explore the extent to which the vernalization pathway is conserved in Pooideae, we characterized the expression and function of vernalization pathway homologs in *B. distachyon* and other pooids.

The current molecular model of vernalization responsiveness in wheat and barley involves a leaf-specific regulatory loop among *VERNALIZATION1* (*VRN1*), *VRN2*, and *VRN3* (Dennis and Peacock, 2009; Distelfeld et al., 2009a; Greenup et al., 2009; Sasani et al., 2009), the latter of which is homologous to Arabidopsis (*Arabidopsis thaliana*) *FT*, which encodes a small protein that moves from leaves to the shoot apical meristem to promote flowering (Yan et al., 2006; Turck et al., 2008). During growth of vernalization-requiring cereals in the fall season, the *CONSTANS*-like gene *VRN2* represses *FT* to prevent flowering, and the *FRUITFULL*-like gene *VRN1* is transcribed at very low levels (Yan et al., 2004a, 2006; Hemming et al., 2008; Sasani et al., 2009). During winter, *VRN1* transcript levels increase, causing the repression of *VRN2* and the derepression of *VRN3/FT* (Yan et al., 2004b; Trevaskis et al., 2006; Sasani et al., 2009). Although vernalization alleviates *FT* repression, *FT* also requires long days to become activated; thus, flowering only occurs during the lengthening days of spring and summer (Yan et al., 2006; Hemming et al., 2008; Sasani et al., 2009). In wheat and barley, *VRN2* is necessary for the vernalization requirement because deletions of the entire locus or point mutations in the CCT domain result in spring varieties, which do not require vernalization (Yan et al., 2004; Dubcovsky et al., 2005; Karsai et al., 2005; von Zitzewitz et al., 2005; Distelfeld et al., 2009b).

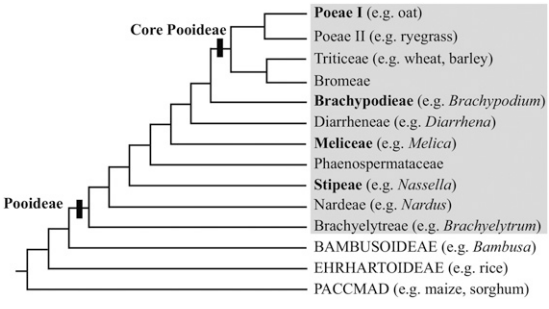


**Figure 1.** Pooideae phylogeny showing the eleven major tribes (gray box) and delimitation of the core pooids based on Schneider et al. (2009) and Grass Phylogeny Working Group II (2012). Outgroups are the closely related grass subfamilies, Bambusoideae and Ehrhartoideae, which together with Pooideae form the BEP clade. Sister to the BEP clade is the PACCMAD clade that contains tropical cereals such as maize and sorghum (*Sorghum bicolor*). Focal tribes in this study are highlighted in bold.

In wheat, there is a negative correlation between *VRN1* and *VRN2* expression in leaves. *VRN1* levels increase during cold and remain elevated following cold (Trevaskis et al., 2003; Yan et al., 2003; Sasani et al., 2009), and this correlates with the stable reduction of *VRN2* during and after cold exposure (Yan et al., 2004). Recently, it was shown that VRN1 binds to the *VRN2* promoter and thus directly regulates *VRN2* expression (Deng et al., 2015). Furthermore, mutations in the wheat *VRN1* locus result in elevated *VRN2* expression and delayed flowering (Chen and Dubcovsky, 2012). The delayed flowering phenotype in the *vrn1* mutants is largely due to the presence of *VRN2* because wheat *vrn1 vrn2* double mutants flower significantly earlier than *vrn1* single mutants (Chen and Dubcovsky, 2012).

Expression of *VRN1* in the noncore pooid *B. distachyon* is consistent with it being conserved as floral promoter involved in vernalization (Ream et al., 2014; for review on flowering in *B. distachyon*, see Woods and Amasino 2015). As in wheat and barley, *B. distachyon VRN1* (*BdVRN1*) mRNA levels increase quantitatively during increasing durations of cold exposure and remain elevated post cold (Ream et al., 2014; Woods et al., 2014). Furthermore, overexpression of *BdVRN1* results in rapid flowering and is correlated with elevated *BdFT* and reduced *BdVRN2* expression (Ream et al., 2014). However, contrary to *VRN2* behavior in core pooids, *BdVRN2* mRNA levels increase rather than decrease during cold, despite a simultaneous increase in *BdVRN1* expression (Ream et al., 2014). Moreover, after cold exposure, *BdVRN2* expression levels return to prevernalization expression levels. Lastly, rapid-flowering accessions of *B. distachyon*, which have elevated *BdVRN1* and *BdFT* mRNA levels without cold exposure, do not have correspondingly lower levels of *BdVRN2* compared to delayed-flowering accessions (Ream et al., 2014). Thus, the *BdVRN2* expression patterns are not consistent with *BdVRN2* acting as a floral repressor that is down regulated by vernalization through *BdVRN1* (Ream et al., 2014).

Here, we conduct extensive phylogenetic analyses that infer a gene duplication event occurred before the divergence of grasses, giving rise to a *CONSTANS9* (*CO9*) and a *VRN2/Ghd7* clade. Analyses across representative pooids suggest that *VRN2/GhD7*-like gene expression is only repressed by vernalization in core pooids, including oats. Furthermore, although functional data in *B. distachyon* demonstrates that *BdVRN2* is indeed a conserved repressor of flowering, *BdVRN1* does not negatively regulate the expression of *BdVRN2*.

Thus, the incorporation of vernalization-mediated repression of *VRN2* as part of the vernalization system was likely to have occurred after the divergence of Brachypodieae and core pooids.

## RESULTS

### Phylogenetic Analyses of *VRN2*-Like Genes Suggest a Duplication Event before the Base of Grasses

Genes from representatives within the cereal grass subfamily Pooideae, in addition to representatives from other grass subfamilies, including Bambusoideae, Panicoideae, and Ehrhartoideae, were included in our phylogenetic analyses of *VRN2*-like genes. Banana (*Musa acuminata*, Musaceae), date palm (*Phoenix dactylifera*, Arecaceae), and oil palm (*Elaeis guineensis*, Arecaceae) genes were also included to sample monocots outside of the grass family. Bayesian and maximum likelihood analyses on an alignment of the highly conserved CCT and 3′ coding domain of *VRN2*-like genes infer two major clades containing cereal *VRN2*- and *CO9*-like genes, respectively (Fig. 2). The best tree topology supports the inclusion of rice (*Oryza sativa*) *Grain number, plant height, and heading date7* (*Ghd7*) and sequences from banana, date palm, and oil palm within the *VRN2* clade (100% posterior probability), which is sister to the supported *CO9* clade (90% posterior probability). The Shimodaira-Hasegawa topology test also supports the position of rice *Ghd7* within the *VRN2* clade (−ln L of 3,554) as the most likely topology; the topologies of *Ghd7* outside of *VRN2* and *CO9*, and *CO9* sister to *Ghd7* were equally less likely (–ln L of 3,562). Together, these data support a gene duplication before the diversification of commelinid monocots, giving rise to the *VRN2/Ghd7* and *CO9* clades. For most Pooideae and Ehrhartoideae (e.g. rice) species sampled, at least two *VRN2*-like genes were isolated, one falling within the *VRN2/Ghd7* clade and the other within the *CO9* clade. Interestingly, two sequences from the banana genome (*M. acuminata*) were

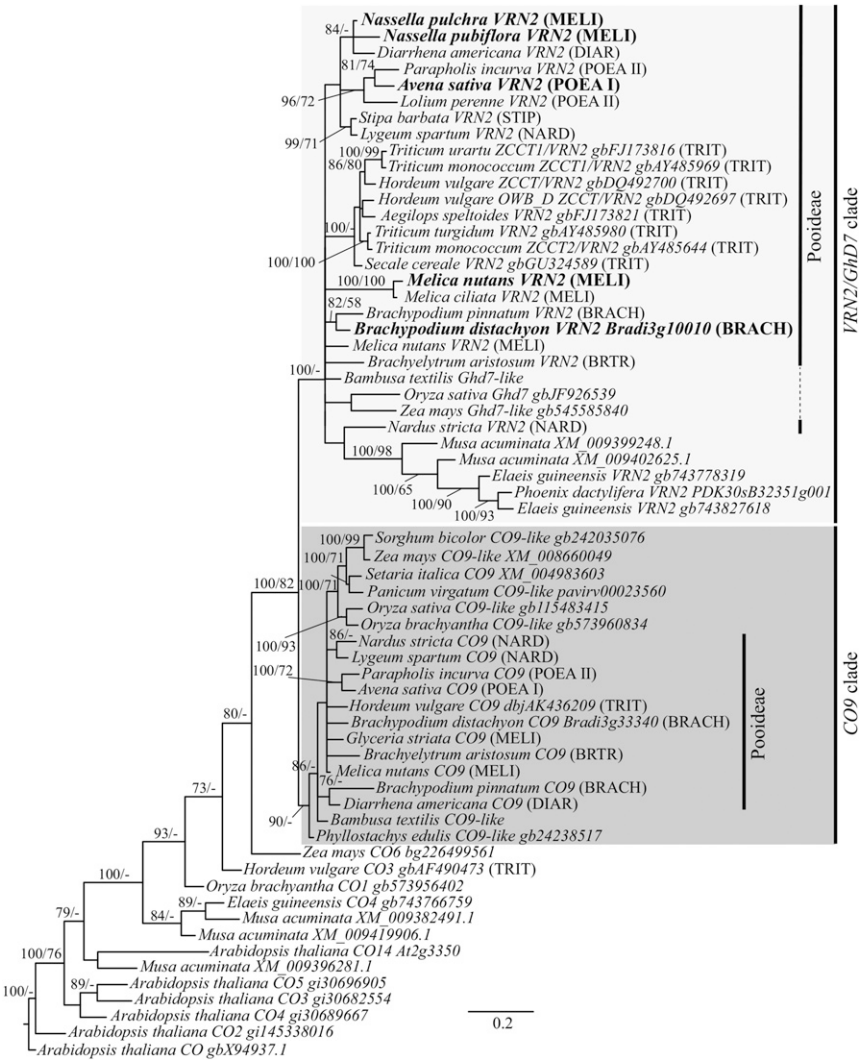


**Figure 2.** Bayesian inference of the phylogenetic relationships among *VRN2*- and *CO9*-like genes and rice *Ghd7* based on a nucleotide alignment of the conserved CCT domain and 3′ coding region. The presence of rice *Ghd7* and *M. acuminata* (wild ancestor of banana) genes in the *VRN2* clade suggests that the gene duplication giving rise to the *VRN2/Ghd7* and *CO9* clades occurred prior to the diversification of commelinid monocots. Bayesian posterior probabilities (left) and maximum likelihood bootstrap (right) support values above 70% are indicated at each branch; dashes denote lower than 70% where applicable. Scale bar indicates substitutions per site. Focal genes are labeled in large bold font. Abbreviated tribal names are indicated for members of Pooideae: BRTR, Brachyelytreae; NARD, Nardeae; STIP, Stipeae; MELI, Meliceae; DIAR, Diarrheneae; BRACH, Brachypodieae; TRIT, Triticeae; Poea I, Poeae I; and Poea II, Poeae II.

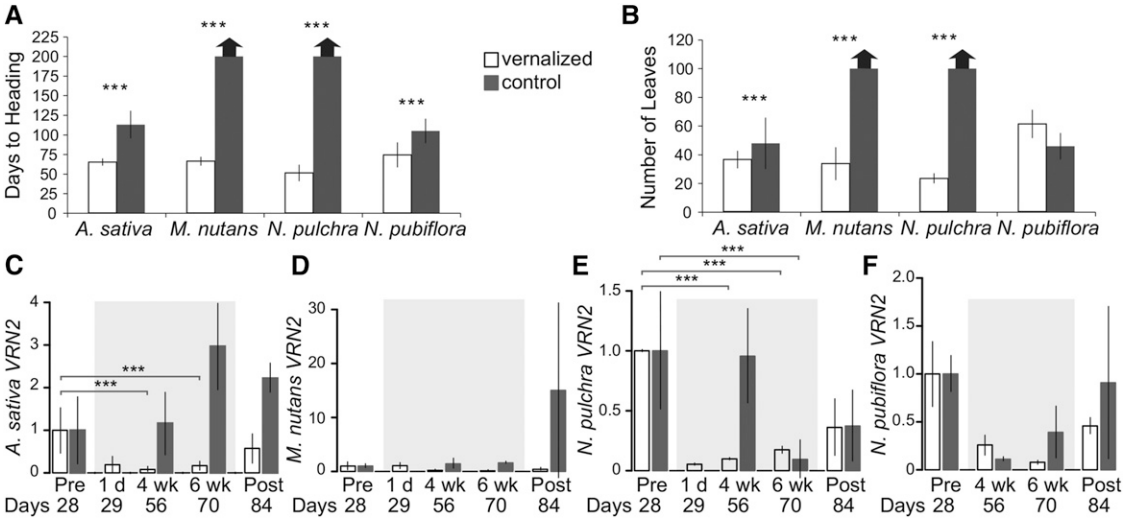


**Figure 3.** *VRN2* regulation in noncore pooids differs from wheat and barley. A, Vernalization causes rapid flowering (days to heading with 6 weeks vernalization subtracted) in *A. sativa*, *M. nutans*, *N. pulchra*, and *N. pubiflora* relative to control conditions. B, Vernalization decreases leaf number at heading in *A. sativa*, *M. nutans*, and *N. pulchra*, but not *N. pubiflora*, relative to control conditions. C, As predicted, winter oat *VRN2* is negatively regulated as a function of cold exposure. D, *M. nutans VRN2* mRNA levels are unaffected by cold and time. E, *N. pulchra VRN2* expression is negatively regulated by time in both cold and control conditions. F, *N. pubiflora VRN2* expression is not significantly affected by cold or time. Thick arrows in A and B denote nonflowering individuals. Error bars in C to F show standard deviations for three biological replicates. Experimental replicates to control for chamber effects showed similar results. Asterisks above bars indicate statistically significant contrasts (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

supported within the *VRN2* clade in addition to oil palm (*E. guineensis*) and date palm (*P. dactylifera*). *CO9* sequences from the banana and palm genomes were not identified, suggesting a loss of *CO9* took place within those lineages. Alternatively, *CO9* sequences are present but were not recovered in the protein blast search. Relationships within each clade did not track the species phylogeny closely, although there was little support for tribal-level relationships. All newly sequenced *VRN2*- and *CO9*-like genes possessed a conserved CCT domain and a conserved nine-amino acid motif upstream of the stop codon. However, the two *Melica nutans* and one *Melica ciliata* (Meliceae) *VRN2* clade genes had a frameshift mutation in the 3′ end of the coding region, leading to a premature stop codon seven amino acids upstream of the usual position. Species used for further analyses were *Nassella pulchra* (Stipeae), *Nassella pubiflora* (Stipeae), *A. sativa* (Poeae I), and *M. nutans* (Meliceae). Members of the Stipeae and Meliceae tribe are the earliest diverging Pooideae representatives included in this study and provided insight into the early diverging lineages of the Pooideae. The Poeae representative (*A. sativa*) gave an expanded picture of the evolution of cold-mediated *VRN2* expression in the core Pooideae. Together, the sampling of different tribes within the Pooideae enabled a diverse look at the evolution of *VRN2* across the Pooideae clade.

### Vernalization Responsiveness Is Widespread in Pooideae

Our data, based on days to heading (after subtraction of six weeks in cold), tiller number at heading, and leaf number at heading, indicate that the core pooid winter oat 'Norline' and the noncore pooids *M. nutans* and *N. pulchra* (Stipeae) are responsive to vernalization. In contrast, although *N. pubiflora* headed sooner in cold than those grown without cold exposure with the conservative subtraction of 6 weeks of the time in cold (Fig. 3A), we consider it nonresponsive to vernalization because it had a similar number of leaves ($P = 0.068$; Fig. 3B) and tillers ($P = 0.017$) in the cold versus warm treatment. Consistent with previous work, winter oat flowered an average of 44 d later without versus with vernalization ($P = 0.025$), with an average of six extra tillers ($P < 0.001$) and 17 extra leaves ($P < 0.001$; Preston and Kellogg, 2008; Fig. 3, A and B). Under warm conditions, *M. nutans* and *N. pulchra* plants failed to flower after 200 d with at least 100 leaves, whereas vernalization resulted in *M. nutans* flowering after an average of 66 d with 10 tillers and 33 leaves, and *N. pulchra* flowering after an average of 51 d with seven tillers and 23 leaves (Fig. 3, A and B). Together with previous studies from *B. distachyon* (Higgins et al., 2010), these results show that vernalization responsiveness is phylogenetically widespread in Pooideae and that there is variation for the presence of this trait in both core and noncore pooids.

### The *VRN2* Expression Pattern during and after Cold Is Different in Core and Noncore Pooids

As predicted based on the model in wheat and barley, *VRN2*-like gene expression in the core pooid winter oat ($P = 0.006$; Fig. 3C) and the noncore pooid *N. pulchra* ($P < 0.001$; Fig. 3E) showed a significant time point by growth temperature interaction. Furthermore, pairwise

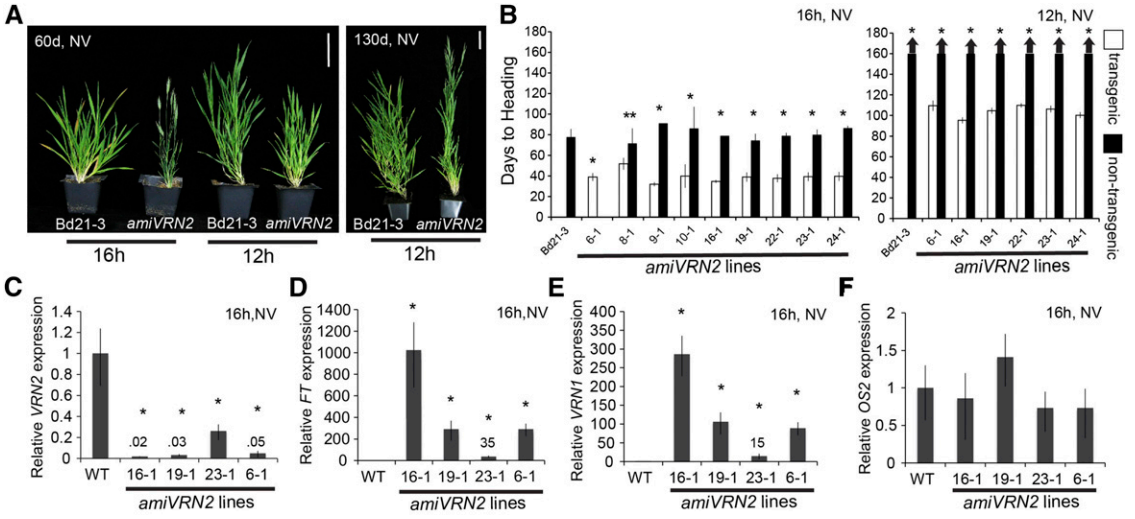


**Figure 4.** *VRN2* knockdown causes rapid flowering. A, Representative photos of Bd21-3 wild-type and rapid flowering *amiVRN2* knockdown plants grown in a 16- or 12-h photoperiods without vernalization (NV). Pictures were taken 60 and 130 d after germination as indicated. Bar = 5 cm. B, Flowering times of Bd21-3 wild-type and segregating nontransgenic (black bars) compared with independent *amiVRN2* transgenic lines (white bars). Lines with no nontransgenic plants are fixed for the transgene. Bars represent the average of 6 plants ± SD. The experiment was repeated with similar results (data not shown). Arrows above bars indicate that none of the plants flowered at the end of the experiment (120 d). C to F, Quantitative RT-PCR expression data from the upper leaf of nonvernalized Bd21-3 wild-type (WT) and *amiVRN2* plants at the three-leaf stage grown in a 16-h photoperiod. Average relative *BdVRN2* (C), *BdFT* (D), *BdVRN1* (E), and *BdOS2* expression (F) is shown for three biological replicates ± SD. Expression analyses were repeated with similar results. Single asterisks indicate *P*-values < 0.01, and two asterisks indicate *P*-values < 0.005. Primers for *BdVRN1*, *BdVRN2*, and *BdFT* were previously optimized by Ream et al. (2014), and *BdOS2* primers were optimized by Ruelens et al. (2013).

contrasts between pretreatment (28 d) and 56 or 70 d time points indicate a significant down-regulation of *VRN2* transcription in winter oat ($P = 0.001$) and *N. pulchra* ($P = 0.001$) with versus without cold (Fig. 3, C and E). However, although expression of *VRN2* in *N. pulchra* dropped after 4 and 6 weeks of cold exposure, a similar decrease in expression was also observed in the warm treatment at the 70 d time point ($P = 0.017$). This suggests that regulation of *VRN2* differs between *N. pulchra* and winter wheat, barley, and oat.

Contrary to predictions, no significant time point by treatment interaction was found for the noncore pooid *M. nutans VRN2* expression ($P = 0.558$; Fig. 3D). Indeed, for this species, *VRN2* expression was similar across both treatments and across all available time points. In the case of the vernalization nonresponsive noncore pooid *N. pubiflora*, we did not expect an effect of time point, treatment, or their interaction on *VRN2* expression; indeed, there was no significant effect of time point by treatment ($P = 0.312$). However, there was a significant effect of time point ($P = 0.002$), with a decrease in expression from 28 to 56 d, followed by an increase from 70 to 84 d in both vernalized and control treatments (Fig. 3F).

### Reduction of *BdVRN2* Expression Results in Rapid Flowering and Elevated Expression of *BdFT* and *BdVRN1*

To investigate *BdVRN2*'s role in flowering time, we transformed the vernalization-responsive *B. distachyon* accession Bd21-3 (Ream et al., 2014) with an artificial microRNA (*amiVRN2*; Warthmann et al., 2008) that posttranscriptionally down-regulates *BdVRN2* mRNA levels. Eight independent transgenic lines segregated for the *amiVRN2* transgene, and one independent line (Bd6-1) had the transgene fixed (Fig. 4B). Under a 16-h photoperiod without vernalization, wild-type Bd21-3 and segregating nontransgenic control plants flowered between 78 and 90 d with an average of 17 leaves (Fig. 4B; leaf data not shown). In contrast, transgenic plants harboring *amiVRN2* flowered between 31 and 52 d and produced an average of five to seven leaves (Fig. 4, A and B; leaf data not shown). This indicates that *BdVRN2* acts as a repressor of flowering under inductive 16-h photoperiods.

In 12- and 8-h photoperiods, wild-type and segregating nontransgenic control plants failed to flower by 160 d without vernalization (Fig. 4B; 8 h data not shown). However, the flowering of the wild type in 12-h photoperiods (but not in 8- or 10-h photoperiods) could be accelerated with 4 weeks vernalization (Supplemental Fig. S1). Even though *amiVRN2* plants flowered more rapidly than the wild type in 12-h photoperiods without vernalization (Fig. 4B), they did not flower as rapidly as 4 week vernalized wild-type plants or lines overexpressing *BdVRN1* (*BdVRN1* overexpression lines flowered around 40 d; Supplemental Fig. S1). Under noninductive 8-h photoperiods, in which *BdVRN2* expression was previously demonstrated to be low in Bd21-3 (Ream et al.,

2014), none of the nontransgenic or transgenic plants flowered after 160 d, consistent with previous findings that 8 h is a noninductive photoperiod (Ream et al., 2014; data not shown). This is consistent with *VRN2* acting as a repressor of flowering only under inductive long days.

As expected, *BdVRN2* expression levels in leaves of four independent *amiVRN2* transgenic lines grown in 16-h photoperiods without vernalization were significantly lower ($P < 0.01$) than expression in wild-type Bd21-3 plants, confirming efficiency of the *amiVRN2* transgene (Fig. 4C). Moreover, expression levels of *BdFT*, *BdVRN1*, and the paralog of *BdVRN1* (*BdFUL2*) were proportionally significantly elevated in leaves of *amiVRN2* transgenic compared with wild-type plants ($P < 0.05$), consistent with their rapid flowering and indicating a role for *BdVRN2* in the repression of *BdFT*, *BdVRN1*, and *BdFUL2* (Fig. 4, D and E; Supplemental Fig. S2). None of the recently identified *FLOWERING LOCUS C*-like (*FLC*-like) genes *ODDSOC1* (*OS1*), *OS2*, and *MADS37* (Ruelens et al., 2013) showed differences in expression in the *amiVRN2* lines compared with the wild type (Fig. 4F; Supplemental Fig. S2). In Arabidopsis, *FLC* is a potent floral repressor turned off by cold conferring a vernalization requirement in Brassicaceae (Amasino, 2010).

### Overexpression of *BdVRN2* Delays Flowering, Resulting in Reduced *BdFT* and *BdVRN1* Expression

To further investigate the role of *VRN2* as a repressor of flowering, we generated 10 independent transgenic Bd21-3 plants constitutively expressing *BdVRN2* under control of the maize (*Zea mays*) ubiquitin promoter. All of the T0 transgenic plants were delayed in flowering compared to control plants lacking the transgene, when grown in a normally highly inductive 20-h photoperiod without prior vernalization (data not shown). For eight of the transgenic lines that segregated for the transgene in the T1 generation, flowering was significantly delayed by roughly 70 d relative to siblings lacking the transgene and wild-type plants in 20-h photoperiods without prior vernalization (Fig. 5, A and B). Furthermore, vernalized (4 weeks) transgenic plants overexpressing *VRN2* flowered roughly 100 d later than vernalized wild-type and nontransgenic sibling plants when grown under inductive 16-h photoperiods (Fig. 5, G and H). Vernalized *UBI:VRN2* lines were even more delayed than nonvernalized Bd21-3 plants; however, they did flower within 120 d, whereas nonvernalized *UBI:VRN2* lines failed to flower and were larger than vernalized plants (Fig. 5, G and H). Thus, overexpression of *BdVRN2* delays flowering and is able to suppress the vernalization response.

As expected, leaf *BdVRN2* expression levels in four independent *UBI:VRN2* transgenic lines grown in 20-h photoperiods were significantly elevated ($P < 0.05$) compared with wild-type Bd21-3 plants (Fig. 5C). Conversely, *BdFT* and *BdVRN1* expression was significantly lower in leaves of *UBI:VRN2* transgenic compared with wild-type plants ($P < 0.05$), consistent with the delayed-flowering phenotype of *UBI:VRN2* plants (Fig. 5, B, D, and E). Interestingly, *BdOS2* expression levels were significantly elevated in the *UBI:VRN2* lines compared with wild-type plants; however, *BdOS1* and *BdMADS37* were unaffected by elevated *BdVRN2* levels (Fig. 5F; data not shown).

*VRN2* expression levels were also significantly elevated in leaves of *UBI:VRN2* lines grown in 16-h photoperiods compared with Bd21-3 either with or without vernalization. The newly expanded third leaf was harvested for both the nonvernalized and vernalized samples when the third leaf was reached. Surprisingly, *VRN2* expression levels were higher in vernalized *UBI:VRN2* lines than nonvernalized *UBI:VRN2* lines (Fig. 5I). As was the case in 20-h photoperiods without vernalization, *BdFT* and *BdVRN1* were significantly lower in the *UBI:VRN2* lines regardless of vernalization treatment, consistent with the delayed-flowering phenotype of the *UBI:VRN2* lines (Fig. 5, J and K). However, *BdVRN1* expression levels were still elevated in the *UBI:VRN2* vernalized lines compared with nonvernalized *UBI:VRN2* lines (Fig. 5K).

### Reduction of *BdVRN1* Expression Results in Delayed Flowering but Does Not Affect the Expression of *BdVRN2*

To evaluate if *BdVRN1* and/or *BdFUL2* (a paralog of *VRN1*; Preston and Kellogg, 2006) affect the expression of *BdVRN2*, and to better define their roles in flowering, we developed artificial microRNAs (amiRNAs) to silence both *BdVRN1* and *BdFUL2* or *BdVRN1* alone (Fig. 6, A and B). Of the eight *BdVRN1* and 18 *BdVRN1/FUL2* independent T0 transgenic lines, none flowered within 220 d when grown in inductive 20-h photoperiods without vernalization (data not shown). Furthermore, seven segregating and one fixed T1 independent transgenic line flowered more than 100 d later than sibling plants lacking the transgene and wild-type plants with 20-h photoperiods (Fig. 6, A and B). However, there was no significant difference in flowering time between *BdVRN1* and *BdVRN1/FUL2* plants, suggesting a redundant function for *BdVRN1* and *BdFUL2* in flowering or that *BdFUL2* does not affect flowering. Unlike wild-type plants that flowered after an average of 20 d, *amiVRN1* plants did not respond to vernalization and failed to flower within 120 d (Fig. 6G; Supplemental Fig. S3). The nonvernalized *amiVRN1* plants also failed to flower after 120 d, whereas nonvernalized wild-type plants flowered after 82 d on average (Fig. 6G; Supplemental Fig. S3).

*BdVRN1* and *BdFUL2* transcript levels in leaves of three independent *amiVRN1/FUL2* and two *amiVRN1* only transgenic lines were significantly lower ($P < 0.05$) than wild-type Bd21-3 plants grown in 20-h photoperiods without vernalization (Fig. 6C; Supplemental Fig. S4C). *BdFT* expression was also significantly lower in leaves of *amiVRN1* or *amiVRN1/FUL2* versus wild-type plants consistent with the delayed flowering phenotype of the former (Fig. 6, B and E). Despite the reduction of *BdVRN1* in *amiVRN1* transgenic plants, the expression levels of *BdVRN2* were not significantly different from wild-type plants (Fig. 6D). However, for the *FLC*-like

genes *BdOS2*, but not *BdOS1* and *BdMADS37*, expression was significantly elevated ($P < 0.05$) in *amiVRN1* plants (Fig. 6F; Supplemental Fig. S4). Recently, *VRN1* has been shown to directly bind to the *OS2* promoter in barley (Deng et al., 2015), and *OS2* expression is reduced in barley lines with highly expressed *VRN1*; however, *vrn1* mutants in barley do not effect *OS2* expression before vernalization (Greenup et al., 2010). In contrast with barley, *OS2* levels are elevated in *B. distachyon* with reduced *VRN1* mRNA levels but do not change relative to the wild type when *VRN1* is up-regulated in *amiVRN2* lines (Fig. 4F) or in *UBI:VRN1* lines (data not shown). It will be interesting to determine if *OS2* contributes to the delayed-flowering phenotype of *amiVRN1* by generating *vrn1/os2* double mutants.

To corroborate the results from above that indicate that *VRN1* does not affect *VRN2* expression, we analyzed the expression of both genes in leaves of wild-type and *amiVRN1* plants harvested before, during, and after cold (Fig. 6, H and I). As expected, *BdVRN1* expression was significantly lower ($P < 0.05$) in *amiVRN1* versus wild-type leaves (Fig. 6H). *BdVRN1* expression significantly increased during and after cold in both wild-type and *amiVRN1* plants (Fig. 6H). However, despite the significantly lower *BdVRN1* expression levels in *amiVRN1* lines, *BdVRN2* expression

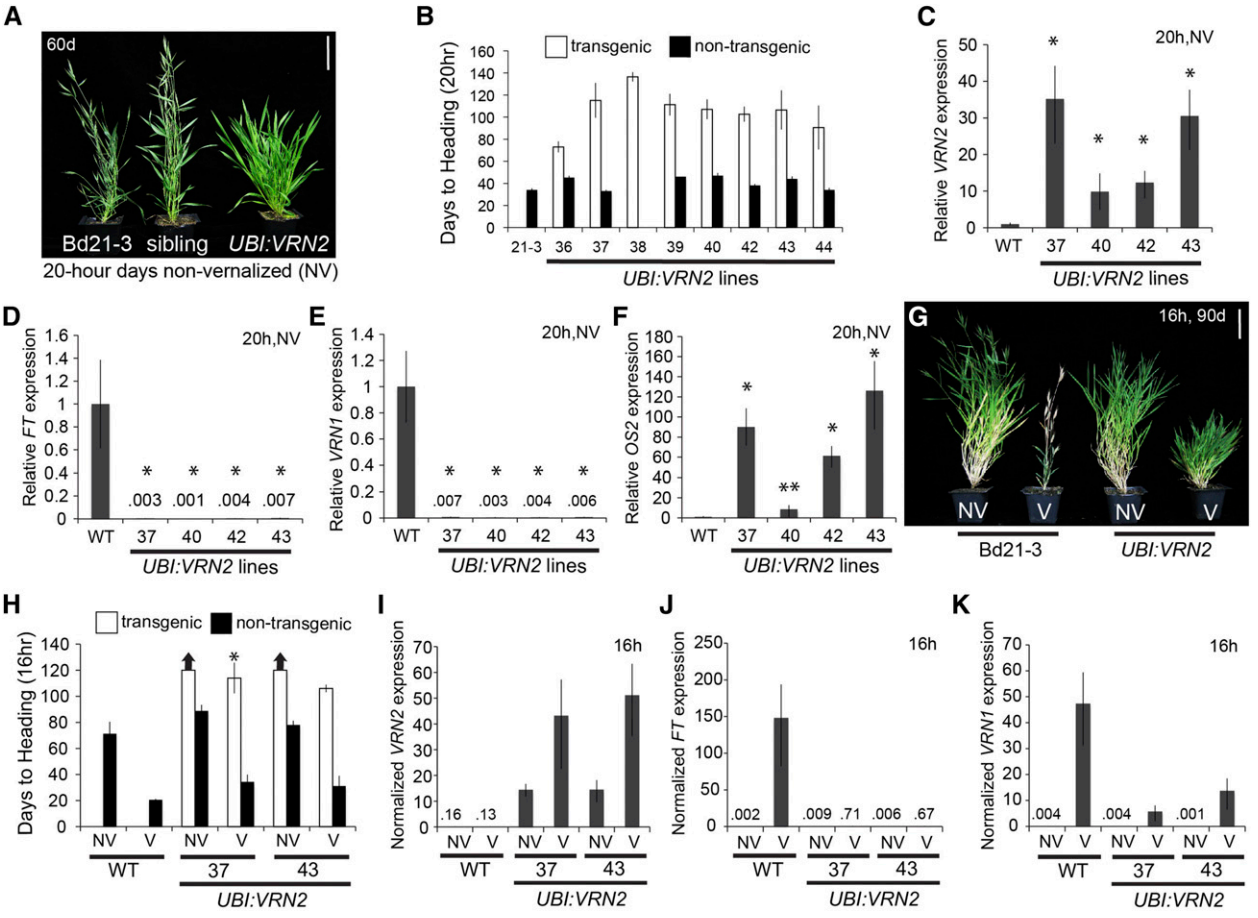


**Figure 5.** *VRN2* overexpression delays flowering. A, Representative photo of Bd21-3 wild-type, nontransgenic sibling, and delayed flowering *UBI:VRN2* plants grown in a 20-h photoperiod without vernalization (NV) 60 d postgermination. Bar = 5 cm. B, Days to heading for Bd21-3 wild type (WT) and segregating nontransgenic (black bars) compared with independent *UBI:VRN2* transgenic (white bars) plants. Lines with no nontransgenic plants are fixed for the transgene. Bars represent the average of six plants ± SD. The experiment was repeated with similar results (data not shown). C to F, Quantitative RT-PCR expression data from the third leaf of Bd21-3 wild type and *UBI:VRN2* plants at the three-leaf stage grown in 20-h photoperiods without vernalization. Average relative *BdVRN2* (C), *BdFT* (D), *BdVRN1* (E), and *BdOS2* expression (F) is shown for three biological replicates ± SD. Expression analyses were repeated with similar results. G, Representative photo of nonvernalized and 4-week 5°C vernalized (V) Bd21-3, and *UBI:VRN2* plants grown for 90 d under a 16-h photoperiod. Bar = 5 cm. H, Days to heading for Bd21-3 wild type and segregating nontransgenic (black bars) compared with independent *UBI:VRN2* transgenic (white bars) plants. Bars represent the average of six plants ± SD. Arrows above bars indicate that none of the plants flowered at the end of the experiment (120 d), and asterisks indicate that only some plants in the treatment did not flower after 120 d. I to K, Average relative qRT-PCR data from the upper leaf of Bd21-3 wild type and *UBI:VRN2* plants at the three-leaf stage grown in a 16-h photoperiod with and without vernalization. *BdVRN2* (I), *BdFT* (J), and *BdVRN1* expression (K) is shown for three biological replicates ± SD.

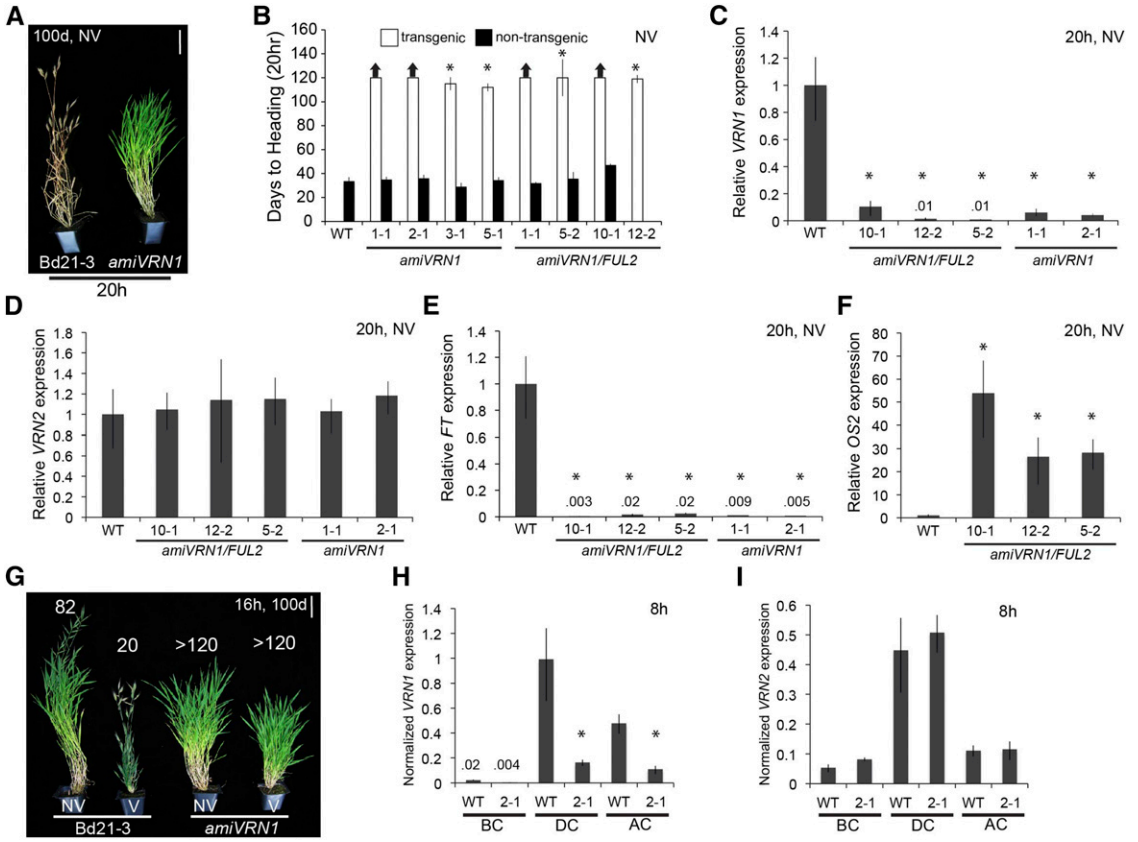


**Figure 6.** *VRN1* knockdown delays flowering. A, Representative image of nonvernalized (NV) Bd21-3 wild type and *amiVRN1* plants grown in a 20-h photoperiod 100 d postgermination. Bar = 5 cm. B, Days to heading for Bd21-3 wild type (WT) and segregating nontransgenic controls (black bars) compared with independent *amiVRN1* transgenic (white bars) plants. Lines with no nontransgenic plants are fixed for the transgene. Bars represent the average of six plants ± SD. The experiment was repeated with similar results. Arrows above bars indicate that none of the plants flowered at the end of the experiment (120 d), and asterisks indicate that only some plants did not flower after 120 d. C to F, Quantitative RT-PCR expression data for the upper leaf of Bd21-3 and *amiVRN1* plants grown in a 20-h photoperiod without vernalization. Average relative *BdVRN1* (C), *BdFT* (D), *BVRN2* (E), and *BdOS2* expression (F) is shown for three biological replicates ± SD. Expression analysis was repeated with similar results. G, Representative photo of Bd21-3 and *amiVRN1* plants grown without or with (V) 4 weeks 5°C vernalization with a 16-h photoperiod. Numbers above plants represent average days to heading of six plants per line (for details, see Supplemental Fig. S3). H and I, qRT-PCR expression data for 8-h photoperiod grown Bd21-3 and *amiVRN1* plants before cold (BC), during 4 weeks cold (DC), and 7 d after cold (AC). Average relative *BdVRN1* (H) and *BdVRN2* expression (I) is shown for three biological replicates ± SD.

was not significantly different from Bd21-3 wild-type plants (Fig. 6I). Similar results were observed in the *amiVRN1/FUL2* transgenic lines (data not shown). Thus, reduction of *BdVRN1* or *BdFUL2* mRNA expression does not affect the expression of *BdVRN2* as is the case in wheat, and *BdVRN2* does not appear to contribute to the delayed-flowering phenotype of the *amiVRN1* or *amiVRN1/FUL2* transgenic lines. However, it will be interesting to determine if *BdVRN2* contributes to the delayed-flowering *amiVRN1* phenotype by generating *vrn1 vrn2* double mutants.

## DISCUSSION

The attainment of flowering competence in response to vernalization has evolved multiple times independently across major lineages of angiosperms and is hypothesized to be a key adaptation facilitating niche shifts from the tropics to the temperate zone (Ream et al., 2012; Preston and Sandve, 2013). One such niche transition occurred in the grass subfamily Pooideae, members of which are distributed primarily in the northern temperate zone (Hartley, 1973; Edwards and Smith, 2009) and are heavily relied upon for grain, turf, and fodder. To determine the likelihood that the known cereal vernalization gene network was established early in the diversification of pooids, we identified vernalization-responsive species outside core Pooideae and tested whether the vernalization-mediated repression of *VRN2* is conserved. Functional analyses in the noncore pooid species *B. distachyon* strongly support conservation of *VRN2* as a repressor of flowering. However, unlike the network in wheat and barley, *BdVRN1* does not negatively regulate *BdVRN2*, and noncore pooid *VRN2* genes are not responsive to vernalization. Together, these data support a model in which orthologous *VRN2/Ghd7* genes have

retained a repressive flowering function during the diversification of pooids, but that co-option of *VRN2* into the network of genes regulated during vernalization occurred after the divergence of Brachypodieae and core Pooideae.

### Evolutionary History of Pooid *VRN2*/*Ghd7* and *CO9* Genes

Two hypotheses about the evolutionary history of *VRN2*-like genes have been previously proposed (Cockram et al., 2012; Ream et al., 2012). One posits a single gene duplication event before the base of grasses, giving rise to a *VRN2/Ghd7*- and a *CO9*-containing clade and implying an orthologous relationship between pooid *VRN2* genes and rice *Ghd7* (Ream et al., 2012). The other postulates two duplication events before the base of grasses, with the first giving rise to the *VRN2* and *Ghd7/CO9* clades and the second producing the *Ghd7* and *CO9* clades, followed by loss of *Ghd7* genes at the base of pooids (Cockram et al., 2012). Our phylogenetic results based on *VRN2/Ghd7/CO9*-like sequences from multiple grass and other monocot species indicate that *VRN2* is orthologous to rice *Ghd7*, supporting a single duplication event before the base of grasses that gave rise to *Ghd7/VRN2* and *CO9* clade genes.

### Conservation of *VRN2* as a Flowering Repressor

Functional data from wheat, barley, *B. distachyon*, and rice, combined with our gene tree topology, strongly support conservation of *VRN2/Ghd7*-clade genes as repressors of flowering under long days. In winter wheat and barley, loss-of-function mutations in *VRN2* are associated with early flowering, and silencing of *VRN2* reduces heading time (Yan et al, 2004a; Dubcovsky et al., 2005). Similarly, we found that *B. distachyon* Bd21-3 ami*VRN2* knockdown and *BdVRN2*-overexpressing lines flower significantly earlier and later than the wild type, respectively. Functional alleles of the rice *VRN2* ortholog *Ghd7* likewise delay heading date under long days (Xue et al., 2008). Similar to many subtropical grasses, rice is a short-day plant, and the requirement for short days to flower in rice is augmented by the long-day repression conferred by *Ghd7* (Xue et al., 2008; Weng et al., 2014). Although little is known about members of the *VRN2/Ghd7* sister *CO9* clade, barley *CO9* has also been shown to prevent precocious flowering, but in this case under noninductive short-day conditions that accompany winter (Kikuchi et al., 2012). Thus, we infer that the ancestor of *VRN2/Ghd7* and *CO9* clades repressed flowering but that photoperiod regulation of these genes evolved following their duplication.

### Co-Option of *VRN2* in Core Pooid Vernalization Responsiveness

Previous work demonstrated that *BdVRN1* and *BdFT* in the noncore pooid *B. distachyon* interact in a positive feedback circuit (Ream et al., 2014), as present in the core pooids wheat and barley (Yan et al., 2006; Shimada et al., 2009; Distelfeld and Dubcovsky, 2010). Although *BdVRN2* appears to act as a repressor of flowering, *VRN1* amiRNA knockdown lines do not show the predicted increase in *VRN2* expression. This indicates that *BdVRN2* does not interact with *BdVRN1*. A second piece of evidence supporting the absence of cold and *VRN1*-mediated regulation of *VRN2* outside core Pooideae comes from gene expression analyses. Rather than decreasing in response to cold, as in winter wheat, barley, and oat (Yan et al., 2004a; Dubcovsky et al., 2005; von Zitzewitz et al., 2005; Distelfeld et al., 2009b) (Fig. 2), *VRN2* expression actually increases transiently in *B. distachyon* Bd21-3 (Ream et al., 2014) or is unaffected by cold in vernalization-responsive *M. nutans* and *N. pulchra*. Thus, despite vernalization responsiveness being widespread throughout subfamily Pooideae, cold-regulated *VRN2* expression appears to have evolved after the major niche transition of Pooideae from the tropics to the temperate zone. This either suggests that pooid vernalization responsiveness evolved multiple times independently or, more likely, that *VRN2* later became subject to *VRN1* regulation, possibly allowing further diversification of core pooids into even colder, more seasonal climates, of the temperate north (Edwards and Smith, 2010).

## MATERIAL AND METHODS

### Plant Growth and Flowering Time Measurements

Seeds of *Parapholis incurva*, winter oat 'Norline', *Brachypodium pinnatum*, *Melica nutans*, *Melica ciliata*, *Stipa barbata*, *Glyceria striata*, *Nassella pubiflora*, *Nassella pulchra*, *Diarrhena americana*, *Lygeum spartum*, *Nardus stricta*, and *Brachyelytrum aristosum* were germinated on 1% agar plates for 1 week in the dark, planted in soil, and grown at 20 to 22°C in long days (16 h light:8 h dark) in a greenhouse at the University of Vermont. *Bambusa textilis* rhizomes were acquired from the U.S. Department of Agriculture (PI 80872) and grown under the same greenhouse conditions. *Brachypodium distachyon* Bd21-3 wild-type and transgenic plants were grown at the University of Wisconsin, Madison as previously described (Ream et al., 2014).

For the flowering-time experiments, at least 40 germinated seedlings of *Avena sativa*, *M. nutans*, *N. pulchra*, and *N. pubiflora* were planted in soil and each individual was randomly assigned to one of two growth treatments. Plants in both treatments were initially grown for 28 d at 20°C, followed by 42 d at 4°C (vernalization treatment) or 42 d at 20°C (control treatment). All plants were then given an additional 14 d at 20°C before being transferred to a common 20 to 22°C greenhouse to monitor for flowering time. Experiments were conducted under long-day photoperiods, and treatments were replicated two or three times. Heading time was measured as days from germination to overtopping of the flag leaf by the inflorescence in warm-treated plants. To correct for inhibitory effects of cold on growth in *A. sativa*, *M. nutans*, *N. pulchra*, and *N. pubiflora*, 6 weeks of cold exposure was not counted in the final heading date. In the case of wild-type and transgenic *B. distachyon* 21-3, seeds were imbibed with water and for vernalization treatments exposed to 5°C for 4 weeks; note that the time in cold was not included in the final heading date as there is limited to no growth during the cold. Nonvernalization temperatures averaged 21°C during the light period and 18°C the dark period.

### Tissue Sampling, RNA Extraction, and cDNA Synthesis

For experiments in *A. sativa*, *M. nutans*, *N. pulchra*, and *N. pubiflora*, leaves for RNA extraction were collected from the youngest expanded leaf of four individuals without repeated measures at 28 (pretreatment), 29 (cold exposure), 56 (4 weeks with or without vernalization), 70 (6 weeks with or without vernalization), and 84 (posttreatment) d postgermination. RNA was extracted using TRI Reagent (Ambion) followed by DNase treatment with Turbo DNA-free

DNase (Ambion) according to the manufacturer's instructions. cDNA was synthesized using 0.5 $\mu$g of RNA in an iScript cDNA synthesis reaction (Bio-Rad). RNA extraction from the upper leaves of *B. distachyon* wild-type and transgenic plants followed Ream et al. (2014).

## Cloning, Sequencing, and Phylogenetic Analysis

*VRN2*-like genes were amplified from leaf-derived cDNA using degenerate primers based on the CCT domain and 3′ coding region of barley (*Hordeum vulgare*) *ZCCT1*, *ZCCT2*, and *CO9*; *B. distachyon VRN2* and *CO9*; and rice (*Oryza sativa*) *Ghd7* and *CO9* (Supplemental Table S1). Longer *VRN2*-like sequences were also obtained from a few species using nested gene-specific forward primers in combination with a polyT reverse primer (Supplemental Table S1). Each amplicon was cloned into pGEM-T (Promega), and eight colonies were picked for Sanger sequencing at Beckman Coulter Genomics. Nucleotide sequences were initially aligned with existing *VRN2*-like genes from GenBank and Phytozome 10.3 using MAFFT (Yan et al., 2003, 2004; von Zitzewitz et al., 2005; Cockram et al., 2007; Pidal et al., 2009; Katoh and Standley, 2013), before manual alignment of amino acid sequences in Mesquite (Maddison and Maddison, 2011; Supplemental Fig. S5). Unalignable regions were pruned from the analysis in order to minimize random noise in the data. A Bayesian analysis was run on the final nucleotide alignment using MrBayes on the CIPRES XSEDE server with 10 million generations, using the GTR+G model as determined by Mr. ModelTest version 2.3 (Ronquist and Huelsenbeck, 2003; Nylander, 2004). Following stationarity, 25% of samples were discarded as burn-in. Maximum likelihood analyses were conducted using RaxML Blackbox on CIPRES XSEDE. The Shimodaira-Hasegawa topology test was done using PAUP4 (Swofford, 2003) on three topologies that were manipulated in Mesquite to differ in the position of rice *Ghd7* (sister to the *VRN2* clade, sister to the *CO9* clade, or sister to both).

## Gene Expression Analyses

*VRN2* qRT-PCR primers for *A. sativa*, *M. nutans*, *N. pulchra*, and *N. pubiflora* were designed in Primer3 (Rozen and Skaletsky, 2000) based on results of our phylogenetic analyses (Supplemental Table S1). Primer efficiencies were checked using the dilution series method (Scoville et al., 2011), and amplicons were sequence-verified. Critical threshold values were normalized using the geomean of two reference housekeeping genes, *UBIQUITIN5* (*UBQ5*) and *ELONGATION FACTOR 1a* (*EF1α*; Supplemental Table S1) as previously described (Scoville et al., 2011). Three technical replicates were used per biological replicate, and three biological replicates were used per two to three experimental replicates for a total of six to nine individual replicates per time point/treatment. For *B. distachyon*, qRT-PCR for *BdVRN1*, *BdVRN2*, *BdFT*, and *BdUBC18* followed Ream et al. (2014) and for *BdOS1*, *BdOS2*, and *BdMADS37* followed Ruelens et al. (2013) with three biological replicates and two experimental replicates. *BdFUL2* primers were optimized as described previously (Supplemental Table S1).

## Statistical Analyses

For *A. sativa*, *M. nutans*, *N. pulchra*, and *N. pubiflora*, linear mixed effects models were employed in R (v3.1.2; multcomp and nlme packages) to test for the effect of time point, treatment, and their interaction on *VRN2* expression. Replicate and time were accounted for as random effects, and data for which there were no a priori predictions (29 d cold shock and 84 d posttreatment time points) were omitted from analyses to reduce heteroscedasticity. Data were subjected to log transformation to increase normality. Pairwise comparisons of expression were done between pretreatment (28 d) and 4 (56 d) or 6 (70 d) weeks of vernalization and between 70 d minus pretreatment expression for vernalization and control treatments. When no time point by treatment interaction was significant, models were simplified by removing the interaction term, and contrasts were done exclusively within time point and treatment. For *B. distachyon*, differences in heading date and gene expression between wild-type, nontransgenic, and transgenic plants were assessed using the Student's *t* test and deemed significant if $P < 0.05$.

## Generation of UBI:VRN2 Transgenic Lines

*BdVRN2* cDNAs were amplified from Bd1-1 cDNA pooled from vernalized and nonvernalized leaf tissue. cDNAs were gel extracted (Qiagen) and were cloned into pENTR-D-TOPO (Life Technologies) using the manufacturer's protocol. Clones were verified by sequencing. pENTR-cDNAs were recombined into pANIC10a (Mann et al., 2012) using Life Technologies LR Clonase II following the manufacturer's protocol. Clones were verified by sequencing in pANIC10a and then transformed into chemically competent *Agrobacterium tumefaciens* strain Agl-1. Plant callus transformation was performed as previously described (Vogel and Hill, 2008). Independent transgenic lines were genotyped for the transgene using a cDNA specific forward and pANIC vector AcV5 tag reverse primer (Supplemental Table S1). Primer pairs used to clone each cDNA are listed in Supplemental Table S1. The *UBI:VRN1* lines were previously published by Ream et al. (2014).

## Generation of amiVRN2 and amiVRN1 Transgenic Lines

amiRNA sequences targeting either *BdVRN2* or *BdVRN1* transcripts were designed using the amiRNA designer tool at wmd3.weigelworld.org based on MIR528 from rice in the pNW55 vector. Parameters were set such that amiRNAs would be specific to the desired target gene, except for DW4 that was designed to target both *BdVRN1* and *BdFUL2*. To increase the chances of obtaining successful knockdown of *BdVRN1* and *BdVRN2* expression, three amiRNAs were developed targeting the 5′, middle, and 3′ ends of the coding region. amiRNAs targeting the 3′ end of both *BdVRN1* and *BdVRN2* were the most efficient for knocking down expression; thus, lines with these constructs were used in all the experiments. Gateway-compatible *amiVRN1* and *amiVRN2* PCR products were recombined into pDONR221 using Life Technologies BP Clonase II following the manufacturer's protocol. Clones were verified by sequencing. The pDONR221 vector containing the desired amiRNA in combination with another vector containing the maize ubiquitin promoter were both recombined into destination vector p24GWI (designed by Devin O'Connor at the Plant Gene Expression Center, Albany, CA) using Life Technologies LR clonase II plus following the manufacturer's protocol. Clones were verified by sequencing to ensure that the maize ubiquitin promoter was upstream from the developed amiRNA in order for the amiRNA to be continually expressed. The generated constructs were transformed into *A. tumefaciens* strain Agl-1. Plant callus transformation was performed as previously described (Vogel and Hill, 2008). Independent transgenic lines were genotyped for the transgene using an amiRNA forward primer specific for the targeted transcript and a reverse primer derived from the pNW55 backbone sequence (Supplemental Table S1). Primers used to generate the amiRNAs are listed in Supplemental Table S1.

## Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers KT354940 to KT354963.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Days to heading of Bd21-3 and OX:VRN1 lines grown in 20,16,15,14,12,10, and 8 hour days.

**Supplemental Figure S2.** BdOS1,BdMADS37 and BdFUL2 gene expression in Bd21-3 and amiVRN2.

**Supplemental Figure S3.** Days to heading of Bd21-3 and amiVRN1 plants non-vernalized and vernalized.

**Supplemental Figure S4.** BdOS1 and BdMADS37 gene expression in amiVRN1 and OXVRN1 and FUL2 expression in amiVRN1/FUL2 lines.

**Supplemental Figure S5.** Alignment of VRN2/Ghd7/CO9 genes and outgroups used for phylogenetic analysis.

**Supplemental Table S1.**

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