Discrete Developmental Roles for Temperate Cereal Grass

VERNALIZATION1/FRUITFULL-Like Genes in Flowering
Competency and the Transition to Flowering1[W][OA]

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Members of the grass subfamily Pooideae are characterized by their adaptation to cool temperate climates. Vernalization is the process whereby flowering is accelerated in response to a prolonged period of cold. Winter cereals are tolerant of low temperatures and flower earlier with vernalization, whereas spring cultivars are intolerant of low temperatures and flower later with vernalization. In the pooid grasses wheat (Triticum monococcum, Triticum aestivum) and barley (Hordeum vulgare), vernalization responsiveness is determined by allelic variation at the VERNALIZATION1 (VRN1) and/or VRN2 loci. To determine whether VRN1, and its paralog FRIUITFULL2 (FUL2), are involved in vernalization requirement across Pooideae, we determined expression profiles for multiple cultivars of oat (Avena sativa) and wheat with and without cold treatment. Our results demonstrate significant up-regulation of VRN1 expression in leaves of winter oat and wheat in response to vernalization; no treatment effect was found for spring or facultative growth habit oat and wheat. Similar cold-dependent patterns of leaf expression were found for FUL2 in winter oat, but not winter wheat, suggesting a redundant qualitative role for these genes in the quantitative induction of flowering competency of oat. These and other data support the hypothesis that VRN1 is a common regulator of vernalization responsiveness within the crown pooids. Finally, we found that up-regulation of VRN1 in vegetative meristems of oat was significantly later than in leaves. This suggests distinct and conserved roles for temperate cereal grass VRN1/FUL-like genes, first, in systemic signaling to induce flowering competency, and second, in meristems to activate genes involved in the floral transition.

The evolution of flowering time matches reproductive activity to suitable environmental conditions for successful production of seeds (Laurie, 1997). Among many temperate species, inflorescence development is quantitatively controlled in response to changes in daylength (photoperiod) and extended periods of cold (vernalization; Loskutov, 2001). Fall-sown winter cultivars are generally tolerant of low temperatures and flower earlier with vernalization, allowing them to delay flowering until the arrival of favorable conditions (Meyer et al., 2004). By contrast, spring cultivars are intolerant of low temperatures and flower later with prolonged periods of cold; facultative cultivars are low-temperature tolerant, but their flowering time is not altered by prolonged periods of cold (von Zitzewitz et al., 2005).

Within the grass family (Poaceae), a large proportion of species adapted to cool climates of both northern and southern hemispheres belong to the subfamily Pooideae (Mejia-Saulés and Bisby, 2000; Grass Phylogeney Working Group, 2001). Pooid grasses comprise approximately 3,300 species (Grass Phylogeny Working Group, 2001), including the economically important cereals wheat (Triticum aestivum; Triticeae), barley (Hordeum vulgare; Triticeae), and oat (Avena sativa; Poeae). Phylogenetic analyses distinguish two groups of pooid grasses: the crown pooids containing Poeae and Triticeae, and the early pooids that are sister to the crown pooids (Davis and Soreng, 2008; Supplemental Fig. S1). Within the crown pooids, many species have been characterized as vernalization responsive (for review, see Heide, 1994). However, both inter- and intraspecific variation for vernalization responsiveness suggests multiple gains and/or losses of this trait. Anecdotal observations (E.A. Kellogg, unpublished data) also indicate that several taxa within the early pooids (e.g., Nardus stricta, Brachypodium pinnatum; Supplemental Fig. S1) might require cold to induce flowering. Because the origins of Pooideae coincide with a shift from tropical to cool temperate regions (Hartley, 1973; Livingstone and Clayton, 1980; Clayton and Renvoize, 1986), it may be hypothesized that vernalization responsiveness represents the ancestral state of pooid grasses. If so, this suggests that spring growth habits evolved multiple times through independent losses of a shared derived vernalization requirement.

In wheat and barley, vernalization responsiveness is determined by epistatic interaction between the genetic loci VERNALIZATION1 (VRN1) and VRN2 (Takahashi and Yasuda, 1971; Laurie et al., 1995; Tranquilli and...
Dubcovsky, 2000). According to the proposed model for vernalization-responsive (winter) growth habits, \( VRN2 \) alleles repress flowering by direct or indirect repression of \( VRN1 \) alleles under long days (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003, 2006; Yan et al., 2003; von Zitzewitz et al., 2005). In crosses between vernalization-responsive (winter) and vernalization-nonresponsive (spring) wheat, the winter allele of \( VRN2 \) is dominant to the spring allele, whereas the winter allele of \( VRN1 \) is recessive (Tranquilli and Dubcovsky, 2000). During vernalization and/or exposure to short days, the transcription of \( VRN2 \) gene products is reduced, resulting in derepression of \( VRN1 \) following a shift back to long days (Yan et al., 2004b; Dubcovsky et al., 2006; Trevaskis et al., 2006). Subsequent up-regulation of genes underlying \( VRN1 \) causes individual plants to become competent to flower (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003, Yan et al., 2003). The transition to flowering generally only occurs after flowering competence has been achieved, following the restoration of warm temperatures and/or long days (Chouard 1960; Yan et al., 2003). However, some winter varieties will eventually flower without vernalization (Karsai et al., 2001).

Several lines of evidence have identified candidate genes underlying \( VRN1 \) and \( VRN2 \) in closely related diploid wheat (\( Triticum monococcum \); Yan et al., 2003, 2004b), hexaploid wheat (\( T. aestivum \); Danyluk et al., 2003; Trevaskis et al., 2003), and barley (Szücs et al., 2006a). Furthermore, quantitative trait loci (QTLs) for vernalization in more distantly related pooid grasses, \( T. turgidum \), map to positions nearly syntenous to the gene of other cereals (Holland et al., 1997, 2002; Danyluk et al., 1998; Jensen et al., 2005; Andersen et al., 2006). In wheat and barley, \( VRN1 \) (also known as \( WAP1 \) and \( BM5 \), respectively) corresponds to one of three \( APETALA1/FRUITFULL (API/FUL)-like MADS-box genes (Schmitz et al., 2000; Yan et al., 2003; Szücs et al., 2006b). Thus, it is nonhomologous to the \( B3 \)-group (Preston and Kellogg, 2006). However, none has explicitly examined patterns of expression in the SAM during the secondary induction phase to determine the potential role of \( VRN1 \) in initiating inflorescence meristem development in perennial ryegrass. Up-regulation of \( LpVRN1 \) in leaves and SAMs in response to vernalization occurs in winter (but not spring) accessions, consistent with a role for this gene in the primary induction of flowering (Petersen et al., 2004, 2006; Andersen et al., 2006). Similar patterns of vernalization- and growth habit-dependent expression have been observed for the closely related \( API/FUL \)-like gene in ryegrass, \( LpMADS2 \) (hereafter \( LpFUL2 \); Petersen et al., 2004, 2006). In Darnel ryegrass (\( Lolium temulentum \)), a vernalization-insensitive ryegrass, \( VRN1 \) and \( FUL2 \) are two of the first genes to be induced in the SAM after exposure to long-day conditions (Gocal et al., 2001). Thus, \( VRN1/FUL2 \) genes may play discrete roles in both flowering competence and the transition to flowering across temperate cereal grasses. To summarize previous studies, expression of \( VRN1 \) has been investigated in leaves and SAMs of winter and spring wheat and leaves of barley. Conversely, expression of both \( VRN1 \) and \( FUL2 \) has been investigated in the SAM of Darnel ryegrass and in leaves and SAMs of perennial ryegrass.
In this study, we investigated multiple oat varieties differing in vernalization responsiveness to establish a link between the potential role of VRN1/FUL genes in the primary and secondary induction of flowering among temperate cereals (Pooidae). We also filled in missing data on the other pooid species by examining FUL2 gene expression in the SAM and leaves of wheat. With these investigations, we now have comparable data on two members of Poeae s.l. (*Avena* and *Lolium*) and two Triticeae (*Hordeum* and *Triticum*; Supplemental Fig. S1), allowing us to test whether the VRN1 response to vernalization is characteristic of at least the crown pooids. The specific objectives of this study were to determine whether, as predicted by the Triticeae model: (1) expression of VRN1 and FUL2 is positively correlated with vernalization treatment in leaves of winter oat; (2) VRN1/FUL2 expression is unaffected or negatively correlated with vernalization treatment in leaves of spring and facultative oat; and (3) up-regulation of VRN1/FUL2 expression in the SAM is correlated with up-regulation in leaves. All experiments were also conducted on appropriate wheat cultivars as positive controls and to determine the precise spatiotemporal pattern of expression in SAMs.

### RESULTS

#### Flowering Time Variation

Of the oat accessions screened, only ‘Norline’ showed a winter phenotype. ‘Clav9014’, ‘Tam Clav9198’, ‘Ogle Clav9401’, ‘Fulghum’, and ‘Wintok’ all flowered significantly later with vernalization and were therefore assigned spring or facultative phenotypes (Table I; Fig. 1). In agreement with Yan et al. (2003), wheat G1777 and PI427802 showed winter and spring phenotypes, respectively. All experiments were carried out under long-day conditions and were averaged for two replicates.

#### Allelic Variation in AsVRN1

Southern-blot hybridization using a probe specific to AsVRN1 revealed three bands corresponding to each genome of hexaploid oat (Fig. 2A). This confirmed that there is a single copy of AsVRN1 in each genome, as reported previously for diploid oat (*Avena strigosa*; Preston and Kellogg, 2007).

To investigate allelic variation at the AsVRN1 locus, we cloned and sequenced AsVRN1 from oat ‘Norline’, ‘Wintok’, ‘Fulghum’, ‘Tam’, and ‘Ogle’ using both genomic DNA and cDNA as templates. Sequences of the C-terminal domain clearly distinguish VRN1 proteins from other members of the gene family. Sequences generated from genomic DNA spanned the C-terminal domain and intron seven, while those generated from cDNA spanned the I, K, and C domains and the 3′-untranslated region (UTR).

Maximum parsimony phylogenetic analysis identified three well-supported clades, presumably corresponding to each of the three oat genomes, each containing a representative of ‘Norline’, ‘Wintok’, ‘Fulghum’ (except clade C), ‘Tam’, and ‘Ogle’ (Supplemental Fig. S2). Sequence identity was 96% between clades A and B, 92% between clades B and C, and 92% between clades A and C. Within the open reading frame, clades A and C had three and 13 SNPs, respectively, distinguishing them from each other and from clade B, whereas within intron seven, clades A and C could be distinguished from each other and from clade B by one SNP plus one indel and 10 SNPs plus one indel, respectively. The single AsVRN1 haplotype from diploid oat was sister to clades B and C together. Although not supported by bootstrap analysis, this placement suggests that clades B and C share a more recent common ancestor than clade A. This is consistent with the proposed evolutionary history of oat species, whereby the hexaploid ancestors of hexaploid oat contained one C and two A genomes, the latter of which evolved to produce the A and D genomes (Li et al., 2000).

In the process of sequencing full-length *AsVRN1* transcripts, we isolated an alternatively spliced form of *AsVRN1* from ‘Wintok’ and ‘Fulghum’. This cDNA consisted of the MADS-box upstream of the first splice site (previously determined for barley and wheat *VRN1*) and a novel 421-bp stretch of sequence leading to the polyT tail. To verify that the novel sequence came from differential processing of intron one, we attempted to amplify the same sequence from RNase-treated genomic DNA of all accessions. Intron one is too long to be amplified easily by PCR, but we were

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**Table 1. Oat flowering time variation in response to vernalization**

Plants were grown under long-day conditions.

<table>
<thead>
<tr>
<th>Oat Cultivar</th>
<th>Days to Flowering, Vernalized</th>
<th>Days to Flowering, Not Vernalized</th>
<th>Growth Habit</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Norline’</td>
<td>86.1 (44.1) ± 11.6</td>
<td>182 ± 31.4</td>
<td>Winter***</td>
</tr>
<tr>
<td>‘Wintok’</td>
<td>96.0 (54.0) ± 12.7</td>
<td>76.8 ± 8.3</td>
<td>Facultative***</td>
</tr>
<tr>
<td>‘Fulghum’</td>
<td>83.7 (47.7) ± 5.9</td>
<td>59.2 ± 7.4</td>
<td>Spring***</td>
</tr>
<tr>
<td>‘Clav9401 Ogle’</td>
<td>67.2 (25.2) ± 11.7</td>
<td>51.8 ± 3.4</td>
<td>Facultative***</td>
</tr>
<tr>
<td>‘Clav9198 Tam’</td>
<td>70.4 (28.4) ± 10.6</td>
<td>59.9 ± 2.1</td>
<td>Facultative***</td>
</tr>
<tr>
<td>‘Clav9014’</td>
<td>76.3 (34.3) ± 12.9</td>
<td>49.2 ± 8.6</td>
<td>Spring***</td>
</tr>
</tbody>
</table>

*SD is shown. Days to flowering minus vernalization treatment is shown in parentheses. Split-plot ANOVA significance levels comparing total days to flowering between treatments: *** = P < 0.001.*
able to sequence a 5’ portion for comparison to the transcript. Sequences from genomic DNA matched those of the cDNA in ‘Wintok’ and ‘Fulghum’, and we isolated the same intronic region from ‘Norline’, ‘Tam’, and ‘Ogle’ (Fig. 2B).

Because deletions in intron one have been implicated in differential regulation of VRN1 in winter and spring accessions of barley (von Zitzewitz et al., 2005; Szu¨ cs et al., 2006b), diploid wheat, T. turgidum, and hexaploid wheat (Fu et al., 2005), we compared the partial intron one sequences of oat with complete intron one sequences of other cereals (Fig. 2B). The partial intron one sequences of ‘Norline’, ‘Wintok’, and ‘Fulghum’ were nearly identical, with only three SNPs identified, one each for ‘Norline’ haplotypes one and two and ‘Wintok’ copy A. Sequences from ‘Tam’ and ‘Ogle’ were identical to each other but shared only 90% identity with other partial intron one sequences from oat. Sequence comparison revealed several regions of significant sequence similarity between oat, barley (Morex), hexaploid wheat (Triple Dirk C), and T. turgidum (Langdon; Fig. 2B). These regions of similarity span the first 454 bp of Morex intron one, approximately 1 kb upstream of the vernalization critical region (von Zitzewitz et al., 2005; Szu¨ cs et al., 2006b).

**Figure 1.** Oat meristems after 6 weeks in the greenhouse at ambient temperatures. A, Meristem of ‘Norline’ at vegetative stage zero. The meristem is smooth and below the flag leaf. B, Meristem of ‘Wintok’ following the transition to flowering. Branch and spikelet primordia are clearly visible. C, Meristem of ‘Fulghum’ at spikelet stage. Spikelets at the top of the inflorescence are further developed than those at the base.


description of VRN1 in leaves is regulated by vernalization in winter, but not spring or facultative accessions of oat (Figs. 3 and 4). The same pattern was also observed for winter and spring accessions of wheat, confirming the results of Yan et al. (2003; Figs. 3 and 4).

In the winter oat ‘Norline’, AsVRN1 expression started to increase after transfer to vernalization conditions in the growth chamber and peaked after 6 weeks of vernalization (Fig. 3). Two weeks after transfer back to greenhouse conditions, AsVRN1 transcription had decreased significantly, almost down to prevernalization levels (Fig. 3). At this stage, meristems were still at vegetative stage zero according to the developmental scale of Gardner et al. (1985) developed for wheat (Fig. 1). In contrast, AsVRN1 was not up-regulated at any time during the control trials, mostly remaining below levels of RT-PCR detection (Fig. 3).

As predicted, spring and facultative oat accessions ‘Ogle’, ‘Clav9014’, ‘Fulghum’, ‘Tam’, and ‘Wintok’ showed AsVRN1 expression profiles that were not positively regulated by vernalization (Fig. 4). AsVRN1 transcripts were detectable in leaves during both vernalization and control treatments. At the pretreatment time point, SAMs of 2-week-old seedlings were vegetative for all accessions (Fig. 4; Supplemental Fig. S3). This corresponded with AsVRN1 mRNA expression in leaves of ‘Ogle’ and ‘Fulghum’ but no detectable expression in leaves of ‘Clav9014’, ‘Tam’, or ‘Wintok’.

**Regulation of Leaf FUL2 Gene Expression by Vernalization**

RT-PCR showed that transcription of AsFUL2 in leaves is regulated by vernalization in winter, but not spring or facultative, accessions of oat (Figs. 3 and 4). Expression of AsFUL2 in vernalized winter ‘Norline’ increased from weeks 4 to 8 and continued to increase after plants were transferred to the greenhouse (Fig. 3). In general, AsFUL2 transcripts were undetectable at all time points during the control treatments (Fig. 3). In contrast, there was no significant difference between treatments on AsFUL2 expression in spring and facultative ‘Ogle’, ‘Clav9014’, ‘Fulghum’, ‘Tam’, and ‘Wintok’ (Fig. 4); AsFUL2 transcripts were amplified from both vernalized and control treatments. In all cases, there was no detectable expression pretreatment and the highest level of expression occurred at week 8 (Fig. 4). Results were similar for two independent experiments.

In winter and spring wheat, there was no significant treatment effect on TmFUL2 expression (Figs. 3 and 4). In winter wheat, gene transcripts were only detectable at week 10, while in spring wheat they were detectable by week 8. These data suggest TmFUL2 is not regulated by vernalization regardless of growth habit.

**Regulation of Meristem VRN1/FUL2 Gene Expression by Vernalization**

To determine the exact timing and location of VRN1 and FUL2 expression within the SAM of wheat and oat
in response to vernalization, we carried out in situ hybridization using gene-specific mRNA probes at 0 to 3 weeks posttreatment for the vernalization trials and at comparable time points for the control trials. Results were reproducible between individual meristems and two independent experiments, and there was little or no hybridization with sense control probes (Figs. 5, I and J and 6, I and J; Supplemental Fig. S3).

In vernalized winter oats (‘Norline’), up-regulation of AsVRN1 expression in SAMs significantly lagged behind the peak of expression observed in week 8 leaves (Figs. 3 and 5). In contrast, expression of AsFUL2 in SAMs was correlated with the peak of expression observed in week 10 leaves (Figs. 3 and 5). Immediately following 6 weeks of vernalization, AsVRN1 and AsFUL2 transcripts were undetectable in vegetative stage zero apices (Fig. 5, A and B), only becoming detectable 2 weeks posttreatment (Fig. 5, C and D), approximately 1 week prior to development of inflorescence branch primordia (data not shown). Unvernalized control winter oats showed no up-regulation of AsVRN1 (Fig. 5E) or AsFUL2 (Fig. 5F) expression in SAMs at any comparable time point.

AsVRN1 and AsFUL2 mRNA was undetectable in vegetative stage zero SAMs of all 2-week-old spring and facultative oat accessions (Supplemental Fig. S3). Sense controls at comparable stages showed background levels of nonspecific staining of nuclei, and
there was no obvious difference in staining between sense- and antisense-treated sections. At the corresponding time point in leaves, transcripts of both genes were undetectable in ‘Clav9014’ and ‘Tam’, but levels of AsVRN1 were already high in ‘Ogle’ and ‘Fulghum’ (Fig. 4). Immediately following 6 weeks in the 20°C growth chamber (unvernalized), ‘Tam’, ‘Ogle’, and ‘Wintok’ were all at spikelet stage. However, in 5-week-old meristems, following only 3 weeks in the 20°C growth chamber, ‘Tam’ meristems were at transitional stage one, whereas ‘Ogle’ spikelets were fully developed. In ‘Tam’ transitional meristems, both AsVRN1 and AsFUL2 were expressed at the apex (Supplemental Fig. S3). In vernalized plants immediately following treatment, AsVRN1 and AsFUL2 transcripts were abundantly expressed in inflorescence branch stage meristems of ‘Ogle’ (Fig. 5, G and H) and transitional meristems of ‘Tam’ (Supplemental Fig. S3). In the vernalization treatment, both ‘Tam’ and ‘Ogle’ had fully transitioned to flowering after 1 week in the cold. In contrast, ‘Wintok’ took much longer to transition to flowering. After 6 weeks cold treatment, ‘Wintok’ meristems were still at vegetative stage zero. However, both AsVRN1 and AsFUL2 were abundantly expressed in the apex of the SAM (data not shown).

In winter wheat, TmVRN1 and TmFUL2 mRNA was abundantly expressed at the apex of the SAM and surrounding leaf primordia immediately after 6 weeks vernalization (Fig. 6, A and B). At this time, all meristems were at transitional stage one according to the developmental scale of Gardner et al. (1985). One week later, individuals had transitioned to late single-ridge stage three or double-ridge stage four (Gardner et al., 1985), the latter characterized by the appearance of spikelet primordia above bract primordia. Two weeks following vernalization, TmVRN1 and TmFUL2 continued to be expressed at the apex of the inflorescence meristem and in developing spikelet meristems on the inflorescence flanks (Fig. 6, C and D). In contrast, no expression of TmVRN1 was detected in SAMs of control winter wheat plants at any time point (Fig. 6E), although TmFUL2 was detectable at low levels in 8- (Fig. 6F) and 11-week-old (data not shown) SAMs. Eleven-week-old control meristems were at transitional stage one.
Unlike winter wheat, TmVRN1 and TmFUL2 mRNA in spring wheat was apparent in 8-week-old SAMs and surrounding leaves of control plants (Fig. 6, G and H). At this time point, meristems were at transitional stage two, but a week later, most individuals had reached the double-ridge stage. Vernalized spring wheat plants transitioned to flowering 2 weeks posttreatment and were at transitional stage one immediately following treatment (data not shown). Expression of TmVRN1 and TmFUL2 was identical to that observed for vernalized winter wheat plants (data not shown), i.e. both gene transcripts were detectable in week 8 apices and in double-ridge stage inflorescence meristems.

**DISCUSSION**

**AsVRN1 and AsFUL2 Are Involved in the Vernalization Pathway of Oat**

Oat AsVRN1 is the ortholog of WAP1 and BM5 (Preston and Kellogg, 2006), two genes involved in the vernalization pathway of winter wheat and barley, respectively (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003, 2006; Yan et al., 2003; von Zitzewitz et al., 2005). To test whether AsVRN1 has a similar role in determining vernalization responsiveness in oat, we carried out RT-PCR of AsVRN1 using RNA extracts from leaves. Consistent with the wheat and barley genetic models, AsVRN1 expression in the winter accession ‘Norline’ only increased during inductive (cold) conditions, and expression was highest prior to the onset of flowering. Under noninductive (warm) conditions, AsVRN1 remained below the level of detection. Expression in spring/facultative accessions was not dependent upon vernalization. Instead, AsVRN1 was expressed constitutively (‘Ogle’ and ‘Fulghum’) or up-regulated in an age-dependent manner (‘Clav9014’, ‘Tam’, and ‘Wintok’). We found no evidence that AsVRN1 of spring/facultative oat cultivars was induced by short-term cold conditions, as has previously been reported for the spring barley ‘Golden Promise’ (Trevaskis et al., 2007). In contrast, AsVRN1 expression appeared to have been delayed or even repressed at the beginning of the cold treatment in oat ‘Ogle’ and ‘Clav9014’ (Fig. 4).

Grasses have another AP1/FUL-like gene (FUL2) that may be partially redundant in function with VRN1 (Gocal et al., 2001; Petersen et al., 2004; Preston and Kellogg, 2006). In vernalization-responsive perennial ryegrass, both LpMADS1 (VRN1) and LpMADS2 (FUL2) are up-regulated in response to vernalization

![Figure 5](image-url). Expression of AsVRN1 and AsFUL2 mRNA in oat meristems determined by in situ hybridization. A representative result is shown. A, Lack of AsVRN1 expression in an 8-week-old winter ‘Norline’ SAM after 6 weeks vernalization (8 week-V). B, Lack of AsFUL2 expression in an 8-week-old winter ‘Norline’ SAM after 6 weeks vernalization (8 week-V). C, AsVRN1 expression in a 10-week-old winter ‘Norline’ SAM after 6 weeks vernalization and 2 weeks in the greenhouse (10 week-V). D, AsFUL2 expression in a 10-week-old winter ‘Norline’ SAM after 6 weeks vernalization and 2 weeks in the greenhouse (10 week-V). E, Lack of AsVRN1 expression in a 10-week-old control winter

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However, the transcription level of LpMADS2 is much lower than that of LpMADS1. Our experiments on oat and wheat FUL2 expression showed a similar trend. Leaf expression of AsFUL2 was similar to AsVRN1, suggesting a qualitatively redundant role for AsFUL2 in the vernalization pathway of oat. In wheat, TmFUL2 expression was not significantly different between winter and spring accessions or between treatments. Thus, TmFUL2 does not appear to have a redundant role with TmVRN1 in the quantitative response to achieving flowering competence. This is further supported by the fact that diploid wheat bearing a nonfunctional TmVRN1 gene remains in the vegetative phase indefinitely (Shitsukawa et al., 2007).

Oat Growth Habit Is Due to Differences in Regulation of AsVRN1/AsFUL2

In wheat and barley, QTLs underlying differences in vernalization responsiveness have been linked to allelic variation in the promoter region or first intron of VRN1 genes. Thus, differences in the regulation of VRN1 genes determine flowering time under certain environmental conditions. Similar studies in hexaploid oat have identified QTLs that map to positions nearly syntenous to the VRN1 gene of other cereals (Holland et al., 1997, 2002; Dubcovsky et al., 1998). This suggests that independent mutations in AsVRN1 may underlie the spring phenotype in oat.

Results from our experiment, examining gene expression under different cold treatments, are consistent with differential regulation of AsVRN1 and AsFUL2 between winter and spring/facultative lines in response to vernalization. However, it is still unknown whether the difference in AsVRN1/AsFUL2 regulation is a consequence of changes in the cis-regulatory elements of these genes or to changes in trans-, in their upstream regulators. As a first step to address this question, we carried out sequence analyses of the AsVRN1 open reading frame, partial intron one, and intron seven, and identified potentially useful SNPs and indels to be used in future QTL studies. We were unable to amplify intron one from winter and spring/facultative oats, as would be expected if it were 11 kb long, similar to the introns in VRN1 genes in other species (Fu et al., 2005). This suggests, but does not prove, that there is no substantial deletion in

Figure 6. Expression of TmVRN1 and TmFUL2 mRNA in wheat meristems determined by in situ hybridization. A, TmVRN1 expression in an 8-week-old winter wheat SAM after 6 weeks vernalization and 2 weeks in the greenhouse (10 week-V). B, TmFUL2 expression in an 8-week-old winter wheat SAM after 6 weeks vernalization (8 week-V). C, TmVRN1 expression in a 10-week-old winter wheat SAM after 6 weeks vernalization and 2 weeks in the greenhouse (10 week-V). D, TmFUL2 expression in a 10-week-old winter wheat SAM after 6 weeks vernalization and 2 weeks in the greenhouse (10 week-V). E, Lack of TmVRN1 expression in an 8-week-old control winter wheat SAM (8 week-C). F, TmFUL2 expression in an 8-week-old control winter wheat SAM (8 week-C). G, TmVRN1 expression in an 8-week-old control spring wheat SAM (8 week-C). H, TmFUL2 expression in an 8-week-old control spring wheat SAM (8 week-C). I, Spring wheat hybridized with a TmVRN1 sense control mRNA probe. J, Spring wheat hybridized with a TmFUL2 sense control mRNA probe. Bars = 100 μm.
any of the AsVRN1 alleles. However, the fact that sequence identity was much higher between introns one versus introns seven of oat, wheat, and barley indicates a regulatory role of intron one and/or a functional role for the alternatively spliced form of AsVRN1.

Cereal VRN1/FUL2 Genes May Be Differentially Regulated in Leaves and SAMs

Flowering time in winter cereals depends upon two stages of inductive signals, one leading to competence to flower (e.g. vernalization) and the other (e.g. warm temperatures) leading to the development of an inflorescence from a vegetative SAM. Previous studies suggest VRN1/FUL-like genes have pleiotropic effects in these discrete stages of flowering. For example, in leaves of winter wheat and barley, up-regulation of TmVRN1 and HvVRN1, respectively, makes plants competent to flower (Danyluk et al., 2003; Trevaskis et al., 2003, 2006; Yan et al., 2003), whereas in Darnel ryegrass, expression of HvVRN1 and LtFUL2 in the transitional apex suggests a role for these genes in the production of an inflorescence meristem (Gocal et al., 2001).

For winter oat ‘Norline’, we found that up-regulation of AsVRN1 and AsFUL2 in 8-week-old leaves correlated with attainment of competency to flower through vernalization. However, no corresponding expression was found in vegetative meristems of competent plants. AsVRN1 and AsFUL2 expression in the SAM was only detectable after secondary induction (warm temperatures), just prior to the production of branch meristems, indicating the transition to flowering. In the case of AsFUL2, increased expression in transitional meristems correlated with the peak of expression in leaves. This expression profile is similar to that reported for perennial ryegrass LpMADS2 (Petersen et al., 2004). In contrast, up-regulation of AsVRN1 expression in SAMs occurred after the peak of expression in leaves, signaling the transition to flowering after competence had been achieved. Later expression in SAMs compared to leaves was also found for the spring and facultative oats, ‘Fulghum’ and ‘Ogle’, respectively.

Expression of TmVRN1 in vernalized 8-week-old SAMs of winter wheat plants confirmed and expanded the results of Yan et al. (2003). In the previous study, vernalized, and therefore competent, 8-week-old SAMs were at vegetative stage zero, and their TmVRN1 expression levels were comparable to the low level found in 2-week vernalized leaves. In our study, vernalized SAMs were already at transitional stage one, and TmVRN1 was abundantly expressed both at the apex of transitional meristems and surrounding leaf primordia. Two weeks later, TmVRN1 expression remained high in SAMs, but expression in leaves was reduced. Taken together, these data show up-regulation of expression is significantly later in SAMs than in leaves, suggesting that TmVRN1 may function in the transition to flowering after competence has been achieved.

New data for TmFUL2 found abundant expression in transitional meristems, despite showing very low expression in leaves. Furthermore, in contrast to TmVRN1, TmFUL2 was also expressed at low levels in vegetative meristems of incompetent winter wheat plants. These data suggest that TmFUL2 was not regulated by vernalization in the SAM. As expected, TmFUL2 transcripts were abundant in transitional meristems of spring wheat regardless of treatment, but expression was much higher than in vegetative meristems of incompetent winter wheat plants. This quantitative difference may reflect differences between growth habits or changes during inflorescence meristem development. Because TmFUL2 expression was also higher in transitional as opposed to vegetative meristems of winter wheat, we prefer the latter interpretation. Thus, TmFUL2 expression in meristems actually increased following attainment of flowering competency, again possibly reflecting a secondary role in the transition to flowering.

In combination with other studies, our data provide evidence that cereal VRN1 and FUL2 genes function in both flowering competence and the transition to flowering. Results from oat and wheat demonstrate that expression in the SAM is not simply part of a systemic response to vernalization. Instead, gene expression is up-regulated after flowering competence has been achieved, suggesting that VRN1 and FUL2 are differentially regulated in leaves and apices. Thus, AP1/FUL-like genes may perform discrete roles in flowering competency and the transition to flowering by partitioning when, where, and how much they are expressed.

Implications for the Evolution of Vernalization Responsiveness of Temperate Cereals

The Pooidae represent an evolutionary shift in habitat from tropical to cool temperate, and thus the use of VRN1 in vernalization response could be related to that habitat shift. This hypothesis predicts that all pooids are ancestrally dependent on vernalization and the spring habit represents a repeated loss of the vernalization requirement. To demonstrate this rigorously requires extensive studies of vernalization response in many species of the subfamily. By investigating oats in some detail, and in combination with other studies, we now have data on two members of Poaeae s.l. (Avena and Lolium) and two Triticeae (Hordeum and Triticum). Taken together, these data suggest that the VRN1 response to vernalization characterizes at least the crown pooids (Supplemental Fig. S1).

Several of the early pooid species also appear to require cold to flower. However, this has not been tested rigorously. It would be of some interest in the future to investigate the vernalization requirement of other pooid species, particularly within the early pooids, to determine if the use of VRN1 in vernalization responsiveness correlates with a change in habitat. Furthermore, it would be useful to determine if temperate grasses
outside Pooidae (e.g. within the large PACCMAAD clade containing Panicoidae, Arundinoidae, Centothecoidae, Chloridoideae, Aristidoideae, and Danthonioideae; Sánchez-Ken et al., 2007) have a vernalization requirement, and if so, whether they respond to cold via a different molecular mechanism.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Accessions of diploid wheat (*Triticum monococcum*) and hexaploid oat (*Avena sativa*) were selected to represent different classes of vernalization responsive growth habit. Winter growth habit wheat G1777 and spring habit wheat PI427802 were utilized as controls for VRN1 expression in leaves (based on Yan et al., 2003), to determine VRN1 expression in meristems, and to determine FUL2 expression in both leaves and meristems. wheat G1777 and PI427802 have the same dominant allele at the VRN2 locus; G1777 is homoygous for the recessive *vrn1* allele and PI427802 is homozygous for the dominant *Vrn1* allele (Yan et al., 2003). Six oat cultivars were characterized as winter or spring based on the difference in heading time under 6-week vernalization and control conditions using split-plot ANOVA. These were winter oat ‘Norline’ (Preston 26), spring oat ‘Fulghum’ (Preston 28) and ‘Clav9014’ (Preston 25), and facultative ‘Wintok’ (Preston 27), ‘Tam Clav9198’ (Preston 29), and ‘Ogle Clav9401’ (Preston 30; Table I). All oat accessions were used to determine allelic variation in VRN1, the effect of cold on expression of VRN1 and FUL2, and the timing of expression in the SAM.

Gene expression experiments were based on a split-plot design with two experimental replicates. Trials were carried out during the summer in a single growth chamber at regular intervals, and sampling was based on a randomized design. Plants were first grown for 2 weeks under 16-h-long day conditions at 18°C to 20°C in a greenhouse with supplemental lighting. Two-week-old seedlings were then transferred to a growth chamber with cool-growth habits were defined as accessions that flowered significantly earlier for 15 to 20 individuals per two independent experiments. Heading conditions and grown until destructive tissue harvesting or heading. Heading time, defined as the date at which the inflorescence overtopped the flag leaf, under vernalization and control conditions was determined as the mean of flowering time for 15 to 20 individuals per two independent experiments. Significant treatment effects were calculated using split-plot ANOVA. Winter growth habits were defined as accessions that flowered significantly earlier with vernalization, whereas spring and facultative growth habits were defined as accessions that flowered significantly later with vernalization without or with some degree of cold tolerance, respectively. Seeds were obtained from the U.S. Department of Agriculture Small Grains Collection or from J.B. Holland (U.S. Department of Agriculture-Agricultural Research Service).

**RT-PCR**

Forty plants were grown for each treatment. Leaf material was harvested from the second leaf of five individuals at 2 weeks (pretreatment); additional sets of five plants were sampled at 4 and 8 weeks (during treatment) and 10 weeks (posttreatment). Each experiment was replicated twice. Total RNA was extracted using Trizol solution (Ambion) according to the manufacturer’s instructions. Wheat and oat gene-specific primers were designed to amplify the target genes VRN1 and FUL2, and *Actin* as a constitutive loading control. Primer sequences were PooidVRN1 (5’-CATCCATGCTAAGGT-3’) and AsFVRN1R (5’-CGAAGGGCCCAAA-3’) to amplify AsVRN1, and AsVRN1F-out (5’-TTT-3’) and AsFUL2R-out (5’-GCAGTCTCATCAGCTCAT-3’) to amplify AsFUL2. Sequencing reactions were analyzed on an ABI-377 terminator cycle sequencing protocol (Applied Biosystems) with the plasmid pGEM-T easy vector (Promega). Each reaction was run for two individuals of each accession to account for potential Taq error and multiple alleles within a population. DNA). To assess sequence conservation in the intron region, the first 500 bp of AsVRN1 of intron one was amplified from genomic DNA of ‘Norline’, ‘Wintok’, ‘Fulghum’, ‘Tam’, and ‘Ogle’ using the forward primer ZAP1oneF and reverse primer AsVRN1R-out (5’-ATTACCGTCCTAACCCTTCCACTT-3’) to amplify AsVRN1. Gene expression experiments were based on a split-plot design with two experimental replicates. Trials were carried out during the summer in a single growth chamber at regular intervals, and sampling was based on a randomized design. Plants were first grown for 2 weeks under 16-h-long day conditions at 18°C to 20°C in a greenhouse with supplemental lighting. Two-week-old seedlings were then transferred to a growth chamber with cool-growth habits were defined as accessions that flowered significantly earlier for 15 to 20 individuals per two independent experiments. Significant treatment effects were calculated using split-plot ANOVA. Winter growth habits were defined as accessions that flowered significantly earlier with vernalization, whereas spring and facultative growth habits were defined as accessions that flowered significantly later with vernalization without or with some degree of cold tolerance, respectively. Seeds were obtained from the U.S. Department of Agriculture Small Grains Collection or from J.B. Holland (U.S. Department of Agriculture-Agricultural Research Service).

**In Situ Hybridization**

Apical meristems from different developmental stages of vegetative and inflorescence development were fixed in formaldehyde-acetic acid (47.5% [v/v] ethanol, 5% [v/v] acetic acid, 3.7% [v/v] formaldehyde [Sigma]) using vacuum infiltration. To increase definition of the cell walls, tissue was stained with 1% eosin Y in 95% ethanol and dehydrated into paraffin wax following vacuum infiltration. To increase definition of the cell walls, tissue was stained with 1% eosin Y in 95% ethanol and dehydrated into paraffin wax following Jackson et al. (1994) and Malcomber and Kellogg (2004). Photographs were imported into Adobe Photoshop and adjusted for contrast, brightness, and color balance.

**Southern-Blot Hybridization, Sequencing, and Phylogenetic Analysis**

AsVRN1 copy number was determined by Southern-blot hybridization carried out following Preston and Kellogg (2006). To determine allelic variation within the coding region and 3′-UTR or intron seven, AsVRN1 transcripts from cDNA and genomic DNA of ‘Norline’, ‘Ogle’, ‘Tam’, ‘Wintok’, and ‘Clav9014’ were PCR amplified using a forward primer designed to bind either at the 3′ end of the MADS-box domain (ZAP4F; 5′-ATTCCTCCTCTCTC-3′), or at the 3′ end of the K domain (AP1.1.1, 5′-GAGAA-GCAGAAGGCCCA-3′; genomic DNA) and either a reverse polyT primer with adaptor (5′-CCGATCTCTAGAGGGCCTTCTTTTTTTT TTT-3′; cDNA) or a reverse primer designed to bind at the 3′ end of a C-terminal domain (ZAP1oneR, 5′-GARGKGGTCACCTCACCCTCATC-3′; genomic DNA). To assess sequence conservation in the intron region, the first 500 bp of AsVRN1 of intron one was amplified from genomic DNA of ‘Norline’, ‘Wintok’, ‘Fulghum’, ‘Tam’, and ‘Ogle’ using the forward primer ZAP4F and reverse primer AsVRN1R-out (5′-ATTACCGTCCTAACCCTTCCACTT-3′). This region corresponded to the section of intron one included in the alternatively spliced transcript of AsVRN1 (see “Results”).

Each reaction was run for 30 cycles with an annealing temperature of 55°C on an MJ Research PTC-200 thermocycler (MJ). PCR products were gel purified through a QIAquick spin column (Qiagen) and subcloned into the pGEM-T-easy vector (Promega). Each reaction was run for two individuals of each accession to account for potential Taq error and multiple alleles within a population.

Plasmid DNA for four clones per ligation was isolated through a QIAprep Spin Miniprep column (Qiagen) and sequenced using the Big Dye 3.1 terminator cycle sequencing protocol (Applied Biosystems) with the plasmid primers T7 and SP6. Sequencing reactions were analyzed on an ABI-377 automated DNA sequencer (Applied Biosystems). Double-stranded sequences were aligned and edited in SeqManII (DNASTAR), and base callings with Phred scores (Ewing et al., 1998) below 20 were resequenced.

Nucleotide sequences spanning the C-terminal domain, intron seven, and 3′-UTR of AsVRN1 were manually aligned using MacClade 4 (Maddison and Maddison, 2003) with GenBank sequences for hexaploid wheat TAlVRN1, barley HvVRN1, and Darnel ryegrass LbVRN1. A maximum parsimony phylogenetic analysis was carried out in PAUP 4.0b10 (Swofford, 2001) using an heuristic search with 1,000 random addition sequences, tree bisection/ reconnection branch swapping, and gaps treated as missing data. Full heuristic nonparametric bootstrap analyses (Felsenstein, 1985) were conducted using 1,000 replicates.
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU283089 to EU283110.

Supplemental Data

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

Supplemental Figure S1. Phylogenetic relationships within subfamily Pooidae (cool temperate grasses) following Davis and Soreng (2008).

Supplemental Figure S2. Maximum parsimony phylogenetic tree showing the relationships between AsVRN1 genes from hexaploid oat 'Norline', 'Wintok', 'Fulghum', 'Tam', 'Ogele', and diploid oat.

Supplemental Figure S3. Expression of AsVRN1 and AsFUL2 mRNA in spring/facultative oat meristems determined by in situ hybridization.

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