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Wheat *VIN3*-like PHD finger genes are up-regulated by vernalization

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Abstract The term ‘vernalization’ describes the acceleration of the transition between the vegetative and reproductive stages after exposing plants to an extended period of low temperature. In Arabidopsis, vernalization promotes flowering by silencing the flowering repressor gene *FLOWERING LOCUS C (FLC)*. Mitotically stable repression of *FLC* is the result of chromatin modifications mediated by the Vernalization-INsensitive 3 (*VIN3*) and *VIN3*-Like (*VIL*) proteins. In this study, we identified and characterized three *VIL* genes in diploid wheat (*Triticum monococcum* L.), named *TmVIL1*, *TmVIL2*, and *TmVIL3*. Similar to Arabidopsis *VIN3*, all three wheat *VIL* proteins carry three conserved domains including a plant homeodomain finger motif (PHD), a fibronectin type III domain (FNIII), and a *VIN3* interacting domain (VID). Genetic mapping placed *TmVIL1*, *TmVIL2*, and *TmVIL3* loci in the centromeric regions of chromosome 5, 6, and 1, respectively. The chromosome location of *TmVIL1* is close to that of the vernalization gene *VRN-D5*, but more precise mapping information is required to validate this relationship. Transcription of the wheat *VIL* genes was up-regulated by vernalization, with a peak after 4–6 weeks of cold treatment. When transferred back to warm conditions, transcript levels of the wheat *VIL* genes returned to pre-vernalization levels. In addition, the transcript levels of wheat

VIL genes are affected by photoperiod. This study indicates that wheat *VIL* genes have retained a similar structure and transcriptional regulation as their Arabidopsis *VIN3/VIL* homologues, suggesting that they might have retained some of their functions.

Keywords Wheat · Vernalization · PHD finger · *VIL* · *VIN3*

Abbreviations

dCAPS	Degenerate cleaved amplified polymorphic sequences
FLC	Flowering locus C
FNIII	Fibronectin type III
LD	Long day
PcG	Polycomb-group
PHD	Plant homeodomain
SD	Short day
VID	<i>VIN3</i> interacting domain
<i>VIL</i>	<i>VIN3</i> -like
<i>VIN3</i>	Vernalization-INsensitive 3

Introduction

The timely transition from the vegetative phase to the reproductive phase is a critical developmental process for plant reproductive success. Multiple flowering promotion pathways have evolved to respond to different environmental cues, and to integrate these signals in a coordinated flowering response (Koornneef et al. 1998; Simpson and Dean 2002). Responses to photoperiod and vernalization are two of the most important mechanisms controlling flowering in many plants (Gilbert 1926). The vernalization response, the acceleration of

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flowering by a long exposure to a chilling treatment (Chouard 1960), is particularly important for plants growing in cold regions.

The molecular basis of vernalization has been investigated in detail in *Arabidopsis* by characterizing a set of genes, defined by mutations that reduce the vernalization response (Chandler et al. 1996). In vernalization-responsive *Arabidopsis* ecotypes, the late-flowering habit is the result of the interaction between dominant, late-flowering genes *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino 1999; Sheldon et al. 1999). The MADS-box gene *FLC* is a repressor of flowering, whose transcription is up-regulated by *FRI* to a level that inhibits flowering. Vernalization represses *FLC*, inducing flowering.

Once vernalized, the cells in *Arabidopsis* apices ‘remember’ their vernalized state through multiple mitotic divisions, a hallmark of an epigenetic event (Wolfe and Matzke 1999; Sheldon et al. 2000, 2002). The establishment of this vernalized state, and the mitotically stable repression of *FLC* in *Arabidopsis* require two interacting plant homeodomain (PHD) finger proteins designated VERNALIZATION-INSENSITIVE 3 (*VIN3*) (Sung and Amasino 2004a) and *VIN3*-LIKE 1 (*VIL1*) (Sung et al. 2006). *VIN3* transcripts are detectable only after an effective cold exposure has been reached for vernalization, and are directly correlated with both the duration of the cold treatment and the degree of *FLC* repression (Sung and Amasino 2004a). *VIL1* transcript levels show only a small increase in response to vernalization but a larger response to short days (SD). *VIL1* is required for the repression of *FLC* and the *FLOWERING LOCUS M* (*FLM*); an *FLC* related flowering repressor (Sung et al. 2006).

Plant homeodomain domains, such as the ones observed in *VIN3* and *VIL1*, are often found in proteins involved in chromatin remodeling complexes (Aasland et al. 1995). Chromatin immunoprecipitation (ChIP) studies have shown a direct interaction between *VIN3* and *FLC* chromatin regions that are remodeled during vernalization (Bastow et al. 2004; Sung and Amasino 2004a). The vernalization-mediated *FLC* repression and the corresponding chromatin changes in histone H3 acetylation and dimethylation are not observed in *vin3* (Sung and Amasino 2004a) or *vil1* mutants (Sung et al. 2006). In addition to the functional *Vin3* and *Vil1* alleles required to establish the *FLC* chromatin modification, two additional genes, designated *VRN1* and *VRN2* (Gendall et al. 2001; Levy et al. 2002), are necessary to maintain the repressed *FLC* state when plants are returned to higher temperature conditions after the vernalization treatment.

Arabidopsis VRN1 and *VRN2* genes are different from the wheat and barley vernalization genes with the same names (Yan et al. 2003, 2004a).

In the temperate cereals, the *VRN1* gene is a MADS-box transcription factor closely related to the *Arabidopsis* meristem identity gene *APETALA1* (Yan et al. 2003). A large proportion of the natural allelic variation in vernalization requirement in the temperate cereals is associated with mutations in the promoter and first intron of this gene (Yan et al. 2004b; Fu et al. 2005), a phenomenon that has not been reported in *Arabidopsis*. A more critical difference in the vernalization pathways between these species is the absence of clear homologues of wheat *VRN2* in *Arabidopsis*, and the absence of clear homologues of *FLC* in the temperate cereals (Yan et al. 2004a). The *VRN2* gene from the temperate cereals is a zinc-finger CCT domain gene down-regulated by both vernalization and SDs (Yan et al. 2004a; Dubcovsky et al. 2006).

In spite of these differences, it is currently not known if the temperate cereals and *Arabidopsis* share similar genes in the early steps of their vernalization pathways. To answer this question, we characterized the wheat homologues of the *VIN3* and *VIL1* genes, which are currently the most upstream genes identified in the *Arabidopsis* vernalization pathway. In this study, we characterized three *VIL* genes in diploid wheat, established the relationship between their protein products with *VIN3/VIL* proteins in rice and *Arabidopsis*, determined their chromosome locations, and examined their transcription profiles under different vernalization and photoperiod conditions.

Materials and methods

Plant materials and growth conditions

The lines of diploid wheat (*Triticum monococcum* L., $2n = 14$, genome A^mA^m) analyzed in this study were *T. monococcum* ssp. *monococcum* DV92 (spring growth habit) and *T. monococcum* ssp. *aegilopoides* G3116 (winter growth habit). These two lines are the parents of the segregating population previously used to construct a linkage RFLP map in diploid wheat (Dubcovsky et al. 1996).

For the SD vernalization experiment, G3116 seeds were planted under long day conditions (LD: 16 h of light/8 h of dark) in a greenhouse at non-vernalizing temperatures (20–25°C). Then, 10-week-old plants were transferred to a cold chamber (4°C) under SD conditions (8 h of light at 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ /16 h of dark). After 6 weeks of cold treatment, plants were moved

back to the LD greenhouse. Plant materials were evaluated immediately before transferring the plants to the cold chamber (0 week), every 2 weeks during the vernalization treatment (2, 4, and 6 weeks), and 2 weeks after returning the plants to the greenhouse under LD conditions (2 weeks out).

For the LD vernalization experiment, G3116 seeds were planted in the greenhouse under LD conditions (20–25°C, 16 h of light/8 h of dark). Two-week-old seedlings were transferred to a growth chamber under LD conditions (16°C, 16 h of light at 230 $\mu\text{mol m}^{-2} \text{s}^{-1}$ /8 h of dark). Three weeks later, 5-week-old plants at the six-leaf stage were transferred to a vernalization chamber under LD conditions (4°C, 16 h of light at 147 $\mu\text{mol m}^{-2} \text{s}^{-1}$ /8 h of dark) for 4 weeks. Meanwhile, control plants were maintained under the original growth conditions. Plants were evaluated immediately before the transfer to the cold chamber (0 week), and then 4 weeks later for both the vernalized (4°C) and control plants (16°C).

For the photoperiod response experiment, G3116 seeds were planted in the greenhouse under LD conditions (20–25°C, 16 h of light/8 h of dark). Two-week-old seedlings were transferred to two growth chambers, one under LD (16°C, 16 h of light at 230 $\mu\text{mol m}^{-2} \text{s}^{-1}$ /8 h of dark) conditions, and the other under SD (16°C, 8 h of light at 211 $\mu\text{mol m}^{-2} \text{s}^{-1}$ /16 h of dark) conditions. Three weeks later, the 5-week-old plants at the six-leaf stage were split into two groups: half were maintained under the same growth conditions (control plants), and the other half was transferred to a growth chamber with the opposite photoperiod. After additional 4 weeks, leaf samples were collected for RNA extractions from the plants transferred to opposite photoperiods as well as from control plants.

DNA extraction, gene cloning, and Southern blotting

Genomic DNA was isolated from leaves of single plants by following a previously published procedure (Dvořák et al. 1988). The coding regions of wheat *VIL* genes were initially cloned from cDNA samples from winter plants (G3116) using the primers described in Table 1. The corresponding genomic sequences were subsequently obtained from the spring plants using clones from the *T. monococcum* DV92 BAC library (Lijavetzky et al. 1999). Using the FailSafe™ PCR kit (Epicenter, Madison, WI, USA), the whole fragments delimited by the cloning primers were amplified from isolated BAC clones and sequenced. Flanking sequences were recovered by the inverse PCR technique (Fu et al. 2006). Procedures for Southern blots and hybridizations were performed according to Dubcovsky et al.

(1994). DNA probes used in the hybridization experiments were prepared from purified PCR products amplified with primers described in Table 1.

Genetic mapping

The wheat *VIL* genes were mapped on a population of 74 F_2 plants from the cross DV92 $\times$ G3116 (Dubcovsky et al. 1996). The *TmVIL1* locus was mapped using a Degenerate Cleaved Amplified Polymorphic Sequences (dCAPS) marker (Michaels and Amasino 1998), which used the primer pair TmVIL1/dCAPS/F1 and TaVIL1/R3 (Table 1), followed by digestion with the restriction enzyme *SspI*. The *TmVIL2* locus was mapped using a 3-bp polymorphic indel detected with PCR primers TmVIL2/Ex2F2 and TmVIL2/I2R2 (Table 1). The *TmVIL3* locus was mapped also using a dCAPS marker, with primer pair TmVIL3/F2 and TmVIL3/dCAPS/R1 (Table 1) followed by digestion with the restriction enzyme *RsaI*. PCR amplification conditions included an initial denaturation cycle at 94°C for 5 min, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C (*TmVIL1*), 58°C (*TmVIL2*), or 54°C (*TmVIL3*), and 30 s at 72°C, and a final 10 min extension step at 72°C. Polymorphic fragments were separated using a 6% acrylamide gel. Genetic linkage maps were constructed using Mapmaker Version 3.0 (Lander et al. 1987) using the Kosambi function (Kosambi 1944).

Real-time quantitative PCR

Total RNA was prepared using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) from leaves as described by Yan et al. (2003). First strand cDNA synthesis was performed using the SuperScript™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's instruction. Gene transcript levels were determined using the SYBR® GREEN PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Real-time PCR assays were performed on an ABI PRISM 7000 SDS (Applied Biosystems) in a final volume of 20 μl containing 1 $\times$ SYBR Green PCR Master Mix, 1 μM primers (forward and reverse), and 1 μl cDNA template. After an initial incubation at 95°C for 10 min to activate the AmpliTaq Gold® DNA polymerase, thermal conditions followed 40 cycles of 95°C for 15 s and 60°C for 1 min, and a final dissociation protocol in a temperature gradient from 60 to 95°C.

In all, five target genes, *TmVIL1*, *TmVIL2*, *TmVIL3*, *TmVRN1*, and *TmVRN2*, were investigated. *TmVRN1* was used as reference in the vernalization experiments and *TmVRN2* served as reference for

Table 1 PCR primers used in this study

Function	Primer name	Primer sequence (5' to 3')
PCR cloning		
<i>TmVIL1</i>	TmVIL1/WF2	ATGGAGTCGACCGGAGGA
<i>TmVIL1</i>	TmVIL1/WR2	AGTGTTCCTATCCGGATT
<i>TmVIL2</i>	TmVIL2/WF1	AGCAGCAGAGGAGGAGAGA
<i>TmVIL2</i>	TmVIL2/WR1	GCAGTGACAGCTTCCGTC
<i>TmVIL3</i>	TmVIL3/WF2	AGACATGTCCAAATCTACTCC
<i>TmVIL3</i>	TmVIL3/WR2	GAGTTAGGGAACCTCTTGAATTG
DNA Hybridization		
<i>TmVIL1</i>	TmVIL1/F1	AGGTTGCTCGTCTACTGCCA
<i>TmVIL1</i>	TmVIL1/R1	AGCTTAGTGACAGAAACAGTCC
<i>TmVIL2</i>	TmVIL2/F1	GATCGTTCTTCAGAGGGAGTTG
<i>TmVIL2</i>	TmVIL2/R1	AGGTTTCATGCAGAAAGCCAGAG
<i>TmVIL3</i>	TmVIL3/F1	ATTCACAGATGCTGATGCGACC
<i>TmVIL3</i>	TmVIL3/R1	AGCTCACTGCCGAAACCTACTT
Genetic mapping		
<i>TmVIL1</i>	TmVIL1/dCAPS/F1	GGTGCGCCAGCTGGGTAAAAT
<i>TmVIL1</i>	TmVIL1/R3	TCTCATTTTCGGAGGCACTCT
<i>TmVIL2</i>	TmVIL2/Ex2F2	GAAGCAGCATGATTTGCTCA
<i>TmVIL2</i>	TmVIL2/I2R2	GAATCCCAGGAAAAACAAGCA
<i>TmVIL3</i>	TmVIL3/F2	AGTGCTGTGGTTCTTCTTGC
<i>TmVIL3</i>	TmVIL3/dCAPS/R1	TATTCCAAGTTAGCTACTAGGTA
Real-time PCR		
<i>TmVIL1</i>	TmVIL1/RTF	CTCTAGCAATGGAGAAGGTAGATGACT
<i>TmVIL1</i>	TmVIL1/RTR	AGGCAGTAGGAAGTGTATCAATTTGT
<i>TmVIL2</i>	TmVIL2/RTF	ACTAACTCACAATTTGAACGATCCAA
<i>TmVIL2</i>	TmVIL2/RTR	ACAGTGATAGATGTAGGAGTTATAGTTCAAAC
<i>TmVIL3</i>	TmVIL3/RTF	TGAAACTTTGCTCTCTAGCGATTCT
<i>TmVIL3</i>	TmVIL3/RTR	GCTGGTAATGAACCTCGAACATG
<i>TmVRN1</i>	TmVRN1/RTF	ACTTATGCACGAATCCATTTCTGA
<i>TmVRN1</i>	TmVRN1/RTR	TTCTGCTTCTCCACGAGTTCCT
<i>TmVRN2</i>	TmVRN2/RTF	CCAACACATGGCTCACCTAGTG
<i>TmVRN2</i>	TmVRN2/RTR	CGTGAAATGGCACGATGTG
<i>TmACT</i>	TmACT/RTF	GCCATGTACGTCGCAATTCA
<i>TmACT</i>	TmACT/RTR	AGTCGAGAACGATACCAGTAGTACGA

photoperiod experiment. *TmACT* (*ACTIN*) was used as an endogenous control for data normalization in all experiments. PCR primers used for the expression analysis are listed in Table 1. A standard curve was constructed for each target gene using ten-fold serial dilutions of a cDNA sample. The efficiency of the primers used in this study was 96% or higher. Expression analysis was performed with 12 biological replicates for each treatment in the SD vernalization experiment, and with 14 biological replicates per treatment both in the LD vernalization experiment and the photoperiod response study. To eliminate the effect of unusually large (or low) values, data points that were more than three standard deviations apart from the mean of each treatment were eliminated from the analyses (this criteria resulted in the elimination of two data points in the SD vernalization experiment, one data point in LD vernalization experiment, and one data point in the photoperiod response experiment, respectively). The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) was used to normalize and calibrate transcript values relative to the endogenous controls.

Sequence analysis

Sequences similar to Arabidopsis VIN3 and VIL proteins were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>) using BLASTP and TBLASTN algorithms. Sequences were assembled using the Vector NTI program (Version 9.0.0, Informa, Frederick, MD, USA). Multiple sequence alignments were obtained using the program CLUSTALW Version 1.8 (Thompson et al. 1994). Primers used for regular PCR, dCAPS marker development, and real-time PCR were designed using the PRIMER3 program (Rozen and Skaletsky 2000), the dCAPS Finder 2.0 (Neff et al. 2002), and the Primer Express software (Version 2.0, Applied Biosystems), respectively. The phylogenetic tree was based on the multiple sequence alignment of the three conserved domains and was constructed using the program MEGA 3.1 (Kumar et al. 2004).

The predicted translation product of VIN3/VIL genes was examined for the presence of protein structural features. Conserved protein motifs were

identified by searching the Pfam database Version 18.0 (Bateman et al. 2004). Coiled-coil domains were predicted using the program Coils, Version 2.1 (Lupas et al. 1991). The Nuclear Localization Signals (NLS) were predicted using the PredictNLS program (Cokol et al. 2000), and the sub-cellular localization was predicted using the LOctree program (Nair and Rost 2005).

Results

Cloning of wheat *VIL* genes

Four rice proteins with regions of at least 40% similarity with Arabidopsis proteins *AtVIN3* (At5g57380), *AtVIL1* (At3g24440), *AtVIL2* (At4g30200), and *AtVIL3* (At2g18880) (Sung et al. 2006) were detected by a BLASTP search of the rice genome. All 16 comparisons, between rice and Arabidopsis VIN3/VIL proteins, showed *E*-values between e^{-44} and e^{-118} indicating highly significant similarities. Other rice proteins exhibited short regions of low similarity (lower than 22%) with significantly higher *E*-values ($E > e^{-10}$) and were, therefore, excluded from further analyses. The four rice proteins showing high similarity with the four Arabidopsis genes were designated as rice VIL proteins, and named *OsVIL1* (ABA98812), *OsVIL2* (BAD38062), *OsVIL3* (AAT94000), and *OsVIL4* (BAD03519). A two-letter prefix is used to indicate the source species of the VIN3/VIL genes described in this study (*At*: *Arabidopsis thaliana*, *Os*: *Oryza sativa*, *Tm*: *Triticum monococcum*).

A recent study of the Arabidopsis *VIL* genes by Sung et al. (2006) reports an additional *VIL* gene: *AtVIL4* (At2g18870), which encodes only the distal VIN3 Interacting Domain (VID). Using this additional Arabidopsis *VIL* protein to query the rice genome, the same four *OsVIL1-4* proteins were recovered ($E < e^{-12}$) from the rice genome, suggesting that these four *OsVIL* proteins are likely a complete set of *VIL* proteins present in the rice genome sequence.

The newly identified Arabidopsis and rice VIN3 and *VIL* proteins were then used to explore the wheat EST database using TBLASTN. Sequence assembling of the multiple wheat ESTs, recovered in the previous search, resulted in three full-length gene sequences, which closely corresponded to three of the proteins from rice. PCR primers specific for each of these three genes (Table 1) were used to recover and sequence cDNAs from *T. monococcum* accession G3116. The three diploid wheat genes were named *TmVIL1* (DQ886919), *TmVIL2* (DQ886917), and *TmVIL3*

(DQ886918) based on their similarity with the rice genes. Wheat ESTs corresponding to *TmVIL2* were the most significant matches when *OsVIL4* was used to query the wheat EST database.

To better understand the evolutionary relationship among the different *VIL* proteins, a phylogenetic tree was constructed using the Neighbor-Joining (NJ) method (Fig. 1). The three *TmVIL* proteins showed close relationship with the rice proteins designated with the same number, and those three clusters were supported by 100% bootstrap confidence values. The *OsVIL4* rice protein was more closely related to the wheat and rice *VIL2* proteins (71% bootstrap value). The wheat and rice *VIL1* and *VIL3* proteins formed a higher level cluster that was more closely related to Arabidopsis *AtVIL1* (100% bootstrap value) than to the other Arabidopsis VIN3/VIL proteins. Arabidopsis *AtVIL2* and *AtVIL3* proteins formed a cluster supported by high-bootstrap values (100%), but the relationship between this cluster and the other VIN3/VIL proteins was not supported by high-bootstrap values and should be considered with caution. Similarly, Arabidopsis *AtVIN3* showed no close relationship with the *VIL* proteins.

AtVIL4 (Sung et al. 2006) lacks the PHD and fibronectin type III (FNIII) domains and therefore was excluded from the previous analysis. We constructed a separate NJ tree including *AtVIL4* using only the VID domain, which is conserved across all VIN3/VIL proteins (Table 3). The VID-based phylogenetic tree placed *AtVIL2*, *AtVIL3*, and *AtVIL4* within one cluster supported by a high-bootstrap value (81%), and retained the same groups as Fig. 1 (data not shown).

Fig. 1 Phylogenetic tree of VIN3/VIL proteins based on the simultaneous analysis of the three conserved domains PHD, FNIII, and VID presented in Tables 2, 3 and 4. Multiple sequence alignments of the conserved domains were produced by Clustal W. The neighbor-joining (NJ) tree was constructed using the complete deletion option from MEGA 3.1. Bootstrap values are indicated close to the nodes and are based on 1,000 bootstrap replicates (only bootstrap values higher than 50% are shown)

Genomic organization of wheat *VIL* genes

DNA hybridization of DV92 genomic DNA Southern blots (two restriction enzymes) with probes for the three wheat *VIL* genes indicated that *TmVIL1*, *TmVIL2*, and *TmVIL3* are all single or low-copy genes (Fig. 2). Hybridization of the probe *TmVIL3* with DNA digested by *DraI* resulted in two restriction fragments, due to the presence of a *DraI* restriction site within the *TmVIL3* probe. The same probes were used to screen the complete *T. monococcum* BAC library by DNA hybridization.

The genomic region for each *VIL* gene was completely sequenced by PCR-based walking using selected clones from the DV92 *T. monococcum* BAC library. The genomic sequences of *TmVIL1* from BAC 652J2 (DQ886922), *TmVIL2* from BAC 657C7 (DQ886920), and *TmVIL3* from BAC 655P13 (DQ886921) were deposited in GenBank. Comparisons of these genomic regions with the corresponding cDNAs revealed that the *TmVIL1* and *TmVIL2* have four exons whereas *TmVIL3* has only three (Fig. 3).

Polymorphisms between parental lines DV92 and G3116 were used to map the three wheat *VIL* genes on the *T. monococcum* genetic map (Dubcovsky et al. 1996) using PCR markers (Table 1). The *TmVIL1* locus was mapped completely linked to a large group of loci clustered at the centromeric region of chromosome 5A^m (e.g., *Xcdo749* 5A^mS and *Xabc706* 5A^mL) (Fig. 4). The chromosome arm location of *TmVIL1* is currently unknown. The *TmVIL2* locus was mapped on the centromeric region of the short arm of chromo-

Fig. 2 DNA hybridization of wheat *VIL* genes. Labels for each lane indicate the probe used and the restriction enzymes used to digest the *T. monococcum* DV92 genomic DNA (M = 1-kb DNA ladder)

Fig. 3 Gene structure and domain organization of wheat *VIL* genes. Rectangles represent exons. Lines represent introns. ATG and TAA designate the start codon and stop codon, respectively. Protein motifs and specialized structures are indicated as CC coiled-coil domain, FNIII fibronectin type III domain, NLS nuclear localization signal, PHD plant homeodomain motif, and VID VIN3 interacting domain

some 6A^m, 3.2 cM proximal to RFLP locus *XksuE3* and 2.1 cM distal to the *Xpsr113* marker, which was completely linked to the centromere (Fig. 4). Finally, *TmVIL3* was mapped on chromosome 1A^m, completely linked to RFLP locus *Xpsr1201*, which was previously mapped on the centromeric region of the long arm of chromosome 1A^m (Fig. 4). Interestingly, all three wheat *VIL* genes were mapped to the centromeric regions of the linkage maps.

Orthology between the three *T. monococcum* *VIL* genes and the corresponding rice genes (same gene

Fig. 4 Chromosome location of *TmVIL* genes

number based on the phylogenetic analysis, Fig. 1) was further supported by the mapping results. *OsVIL1* is located on the long arm of rice chromosome 12 (≈ 21.2 Mb), which is co-linear with the centromeric region of wheat homoeologous group 5. *OsVIL2* is located on the short arm of rice chromosome 2 (≈ 2.9 Mb), which is co-linear with wheat chromosome 6, where the *TmVIL2* gene was mapped. Finally, *OsVIL3* is located on the short arm of rice chromosome 5 (≈ 2.6 Mb), which is co-linear with wheat chromosome 1, where *TmVIL3* was mapped.

Analysis of wheat VIL proteins

All three wheat VIL proteins, *TmVIL1* (697 amino acids), *TmVIL2* (750 amino acids), and *TmVIL3* (615 amino acids) include a PHD finger domain. This domain is located at the end of the second exon in *TmVIL1* and *TmVIL2* and at the end of the first exon in *TmVIL3* (Fig. 3). All VIN3/VIL proteins (Table 2) show the conserved Cys₄-His-Cys₃ signature characteristic of the PHD zinc finger domain (Aasland et al. 1995).

The Arabidopsis VIN3/VIL proteins also include a conserved domain at the carboxy end of the proteins, designated VID based on its role on the physical interactions among this group of proteins (Sung et al. 2006). This region was also well conserved in all the wheat and rice VIL proteins included in this study (Fig. 3; Table 3).

A FNIII domain was found between the PHD and VID domains in the Arabidopsis VIN3/VIL1 proteins (Sung et al. 2006). This FNIII domain is less conserved in the grasses (Table 4) than the PHD and VID domains described above. *AtVIN3* and all members of VIL1 and VIL3 proteins showed higher similarity to the pfam FNIII domain than *AtVIL2*, *OsVIL2*, *OsVIL4*, and *TmVIL2* (Table 4).

The LOCtree program predicted a nuclear localization for the three wheat VIL proteins and classified them as DNA-binding proteins. The presence of a putative NLS (TVKRC) in *TmVIL1* provided additional evidence of the nuclear localization of this protein (Fig. 3). A comparison with known coiled-coil proteins showed that both *TmVIL1* and *TmVIL3* contained a protein segment that would likely adopt a coiled-coil conformation (Fig. 3).

Transcription of wheat VIL genes under vernalization treatment

Short day vernalization

All three wheat VIL genes showed a significant up-regulation during the course of vernalization. Significant differences ($P < 0.001$) in transcript levels were detected between the vernalized and un-vernalized plants (Fig. 5a–c). *TmVIL2* and *TmVIL3* showed maximum transcription levels after 4 weeks of vernalization (4w, Fig. 5b, c), whereas the maximum for *TmVIL1* was observed after 6 weeks of vernalization (6w, Fig. 5a).

None of the wheat VIL genes showed significant differences ($P > 0.05$) in transcript levels between the samples collected before vernalization (0w, Fig. 5) and those collected 2 weeks after moving the plants out of the cold room and back to the greenhouse under LD and warm conditions (2wOut, Fig. 5). These results indicate that the increases in *TmVIL* transcript levels observed during the vernalization treatment are not permanent. The *VRN1* gene, which was included as a reference (Fig. 5d), showed the expected up-regulation of transcript levels ($P < 0.001$) that is characteristic of a successful vernalization process (Yan et al. 2003).

Table 2 PHD finger domain alignment

Gene	Acc. No.	PHD domain alignment
<i>AtVIN3</i>	AAR91717.2	CRRCSCCICOKFDDNKDPSLWLTC-----DACGSSSCHLECOLKQDRYGLGSDD---LDG-RFYCAYCGKD
<i>AtVIL2</i>	BAB17836	CRRCSCCICRKVDDNKDPSLWLTCSSPPPFEGSCGFSCHLECAFNTBKSGLGKD---KQSEGCCFYCVSCGKA
<i>AtVIL3</i>	AAX23819	CRRCSCCICFKYDDNKDPSLWLTCNSFSQFEGSCGLSCHLNCAFDSEKSGLKEDTPSSDLDG-CFNCVSCGKT
<i>OsVIL4</i>	BAD03519	CRRCSCCICHKYDENKDKPSLWLVCSSSTPYSGYSGCTSCHLKALKKNKAGLFFKNKCNKNSDGS-SEYCVWCGKM
<i>OsVIL2</i>	BAD38062	CRRCSCCICFKYDDNKDPSLWLTCSSSQPLQKDSVFSCHLECALKDGRGTGMQSGCKNLDDG-GYYCTRCRRQ
<i>TmVIL2</i>	DQ886917	CRRCSCCICFKYDDNKDPTTWLSCSSDHPMQKDSGLSCHLECALKDGRGTGLPSGCKCKLDDG-AYYCPNCRKQ
<i>AtVIL1</i>	NP_189087	CRRCSCCVCHNFDENKDKPSLWLVCPEPKSDVEVFCGLSCHTECAFREVKVGNIALGNLMLDGD-CFCYSCGKV
<i>OsVIL3</i>	AAT94000	CRRCSCCICHQFDDNKDPSLWLVCASE-NDDKNCCGSSCHTECALQHKRVGCFNLGNIIQLDG-SYSCASCCKV
<i>TmVIL3</i>	DQ886918	CRRCSCCICHQFDDNKDPSLWLVCASE-NDDKQCCGSSCHTECALQQRVGCDFDLQKIIHLDD-SYSCASCCKI
<i>OsVIL1</i>	ABA98812	CRRCSCCICHLFDDNKDPSLWLVCSSS-TGDRDCCBSSCHTECALQHKVGCVDLGGSIQLDDG-NYCCAACGKV
<i>TmVIL1</i>	DQ886919	CRRCSCCICHLFDDNKDPSLWLVCSSS-TGDTDCBSSCHVECALQRRKAGRIDLGGSMHLDDG-NYCCAACGKV

Cys₄-His-Cys₃ Signature* --C--C-----C-----C---H--C-----C--C---

* As reported by Aasland et al (1995).

Table 3 VID domain alignment

Gene*	VID domain alignment **
AtVIN3	DLGHIVKTIIRLEEEGHIIKSFRRFLTWYSLRATHREVRVVKIFVETFMEDLSLGGQLVDTFSESILSKRS
AtVIL2	GLDHCVKTIIRLEECGHHIKNFRQKFLTWYSLRATSOEIRVVKIFIDTFIDDEPMALAEQLIDTRDDRVSIKRS
AtVIL3	GEFECVNIIRLEECGQVKSDFRKKFLTWYCLKATDKKHHVVEIFVDTFKDDKEALAKQLIDTFSDCTTRKHE
AtVIL4	GLEQCVKTIIRLEECGIVVESTFRQKFLTWYSLRATSOERNITVTTFMNAFNDDSMALAEQLVDTFSDCTWSKGS
OsVIL4	QYEVSVKVIIRWLEHGHMDKDFRVKFLTWYSLRATSOERRIVNAFVDALVSDPASTVAQLIDSRFMEVVCSEK
OsVIL2	SYEYCVKVVIRWLECGYIETNFRVKFLTWYSLRATPHDKITVSVVNTLIDDEPVSLSGQLADTFSEATYSKRP
TmVIL2	TYEYCVVIRWLETEGYIETNFRVKFLTWYSLRATPHDKITVSVVNTLIDDEPVSLSGQLADTFSEATYSKRP
AtVIL1	TLKCVKVIIRWLEREGHIKITFRVRFLTWYSLRATSOEIRVVKIFVETFMEDLSLGGQLVDTFSESILSKRS
OsVIL3	DEYCVOKIRMLEECGHHIKNFRMKFLTWYSLRSTENRRVVTTFKTLINBPSGLAEQLVDSFGGEATNCKRQ
TmVIL3	DYECVKVIRWLECGHIEIDFRLKFLTWYSLRSTENBHRVNTTFKTLINBPSGLAEQLVDSFGGEATNCKRQ
OsVIL1	NYEYCVKVIIRWLECGHHIBKDFRMKFLTWYSLRSTEQERRVVTTFRTLADDESSLAGQLLDSFEETVSSKKRP
TmVIL1	NYEYCVKTIIRWLECGHIEKFRMRFLTWYSLRSTEQERRVVTTFRTLADDESSLAGQLLDSFEETVSSKKRP

* Acc. Nos. are the same as in Table 2, with the addition of *AtVIL4* (NP_179477.2)

** According to the VID domain alignment reported by Sung et al. (2006).

Table 4 FNIII domain alignment

Gene*	FNIII domain sequence alignment **
AtVIN3	KMT---VRVEEIIQA--RSVTIRVDSEEPSSSTQNKITCFRLR--KSKDEECSSQGNQV--VYLPETTSATQGLEPDTFCLRVVSFNEEGD--IDES
AtVIL2	TTGST-KIRFEDVNATSLVVLAS-NEIPS---PPNIVHYSIWHRKVPEKTYEEKSTCT--LFIPNTRFVVSGLAPASEYCFKVSYSGTRE--MGVDE
AtVIL3	TIQGSMTKRIEISVLATSVIFDTEAEESFSWG---DTNHRYRNVYRKVSEKHS--SKDLTRE--FSTSSHOFVMBELTPATEYWFKIVSFGVBE--LSVDE
OsVIL4	F--CPQPOVFFVEITPFSVLVVEKY-QDN---IAEELDCCKVWHRSANMANYP--ABETCHVLEPNTRSFSGSLSPSTEVFFKVLFPQCSQG-YCEWE
OsVIL2	RMIPSNFVKFEAITQTSVTVVLDL-GPILA---QDVTCENVWHRAVATG---SFSSSETGIIILAPLTLVVTQLVPATSVIFKVNAFSNIYK-FGSWE
TmVIL2	NMPPSSFTKFEPIPTPTSIIVVFDL-ARCPYI--SQGVTEKVVHQQVDGIGFY--SLNETGTVHLMS--TFVVTALKPATCYMIKVTAFSNSSE-FVPWE
AtVIL1	PAA--CRHFEDIAP--KQVTIRL--IELPSAVEYD-VKGYKLWYF--KKGEMPE--DDLFDCSRTERRMVISDLPECTEYTFRVVVSYTEAGI-FCHEN
OsVIL3	PAA--CRFRFVETIS--SSIVLIL-KETP-LPSSDTIRGYKLWY--KSRBPPS-MEBEVV--LSKQDKVIVFNLPATCTQVSFRIISFTDDGI-LGHSE
TmVIL3	PAA--CRFPFEDITS--SSLVVIL-KETK-LASSDTIKGYKLWY--KSRBPPS-MDBEVI--LSKDERKILVYNLATCTEYSFRIISFSDADATIGHSE
OsVIL1	PAA--CRFPFEDITT--SSLVVVL-KEAASS-QYHAIKGYKLWY--NSREBPS-TRVEAI-FPKDQRRILVSNLOPCTEYAFRIISFTEYGD-LGHSE
TmVIL1	PTA--CRFPFEDITA--SSLVVVL-KETASS-QYHAIKGYKLWY--NSREBPS-TGBEVI-FPKDQRRILVSNLOPCTEYAFRIISFVEDGE-LGHSE

* Acc. Nos. are the same as in Table 2.

** FNIII domain from Pfam database (Bateman et al. 2004).

Long day vernalization

This second experiment, using 14 5-week-old plants per treatment, showed a significant up-regulation of the three wheat *VIL* genes after 4 weeks of vernalization (Fig. 6a–c). In all cases, highly significant differences ($P < 0.001$) were observed in the statistical contrast between the vernalized plants (4w ver., Fig. 6) and the average of the un-vernalized plants before vernalization (0w, Fig. 6) and the control plants maintained under warm conditions (4w no v., Fig. 6). Of the three *VIL* wheat genes, the *TmVIL1* transcripts had the most pronounced response to cold (Fig. 6a), a result that was also observed under SD vernalization (Fig. 5).

The contrast between the samples collected before vernalization (0w, Fig. 6) and the control plants maintained under warm conditions (4w no v., Fig. 6) showed no significant differences for *TmVIL1* and *TmVIL3* ($P > 0.05$). However, *TmVIL2* transcript levels were higher ($P < 0.01$) in the un-vernalized samples collected from 9-week-old plants (4w no v., Fig. 6b) relative to the samples collected from un-vernalized 5-week-old plants (0w, Fig. 6b). The *VRN1* gene, which was included as a reference (Fig. 6d), showed signifi-

cantly higher transcript levels ($P < 0.001$) after 4 weeks at 4°C than before this treatment, confirming a successful vernalization process.

Photoperiod response of the transcription of wheat *VIL* genes

To simplify the description of the complex results from this experiment, we will use a two-letter abbreviation for the different treatments where the first letter indicates the photoperiod (SD or LD) during the first three initial weeks and the second letter indicates the photoperiod during the next 4 weeks. For example, the SL treatment indicates plants grown initially under SD and then transfer to LD, whereas SS indicate the control plants that were maintained under SD for the whole duration of the experiment (Fig. 7).

Both *TmVIL1* and *TmVIL2* showed significantly higher transcript levels ($P = 0.04$ and 0.001, respectively) in plants grown initially under SD (SS and SL, grey bars, Fig. 7a, b) than in the plants grown initially under LD (LL and LS, black bars). However, the transfer from SD to LD or from LD to SD after the first 3 weeks had no significant effects on the transcript levels of these two genes ($P > 0.10$).

Fig. 5 Time course of *TmVIL* transcript levels during SD vernalization. Transcript levels of *TmVIL1* (a), *TmVIL2* (b), *TmVIL3* (c), and the vernalization reference *TmVRN1* (d) were estimated by quantitative PCR. The values on the Y-axis represent normalized and calibrated values relative to the endogenous ACTIN control using the $2^{-\Delta\Delta C_T}$ method. Values for each treatment are

averages of 12 10-week-old G3116 plants. RNA samples were extracted from leaves immediately before vernalization (0w) and then every 2 weeks (2w, 4w, and 6w). The final samples were collected 2 weeks after transferring the plants back to warm conditions (2w out)

Fig. 6 Effect of long day vernalization on wheat *VIL* transcript levels. Transcript levels of *TmVIL1* (a), *TmVIL2* (b), *TmVIL3* (c), and the vernalization control *TmVRN1* (d) were estimated by quantitative PCR. The values on the Y-axis represent normalized and calibrated values relative to the endogenous ACTIN control using the $2^{-\Delta\Delta C_T}$ method. Values for each treatment are averages

of 14 5-week-old G3116 plants. RNA samples were extracted from leaves immediately before vernalization (0w) and 4 weeks after vernalization (4w ver.). Control plants were maintained in the chamber under non-vernalization conditions and were measured simultaneously with the plants in the cold chamber (4w no v.)

On the contrary, the transcript levels of *TmVIL3* showed no significant differences ($P = 0.48$) between plants grown initially under SD and LD (Fig. 7c), but highly significant increases ($P < 0.001$) in the plants transferred from LD to SD (SL) relative to the LD controls (LL). A favorable effect of SD on *TmVIL3* transcript levels was also observed on the plants grown initially under SD. Plants maintained under continuous SD (SS) showed slightly higher *TmVIL3* transcript levels than those moved to LD (SL)

(Fig. 7c). However, the differences were not significant ($P = 0.18$).

The photoperiod responsive gene *TmVRN2* was measured as a reference to evaluate the effect of the conditions in each growth chamber. In agreement with a previous study (Dubcovsky et al. 2006), SD had a significant effect in reducing *TmVRN2* transcript levels ($P < 0.001$) (LL versus LS and SS versus SL, Fig. 7d). On average, plants grown initially under SD showed 30% lower *TmVRN2* transcript levels than those

Fig. 7 Quantitative PCR determination of the effect of photoperiod on wheat *VIL* transcript levels. Transcript levels of *TmVIL1* (a), *TmVIL2* (b), *TmVIL3* (c), and *TmVRN2* (d). The photoperiod sensitive *TmVRN2* gene (Dubcovsky et al. 2006) was added as reference. The values on the Y-axis represent normalized and calibrated values relative to the endogenous ACTIN control us-

ing the $2^{-\Delta\Delta C_T}$ method. Values for each treatment are averages of 14 9-week-old G3116 plants. RNA samples were extracted from leaves corresponding to four different treatments: LL = 7-week LD, LS = 3-week LD followed by 4-week SD, SS = 7-week SD, and SL = 3-week SD followed by 4-week LD

grown initially under LD ($P < 0.001$). The *TmVRN2* transcription data confirmed that the plants in this experiment showed the expected SD and LD responses for other known photoperiod responsive genes.

Discussion

VIL map location and relationship to loci affecting vernalization

In temperate cereals, the vernalization requirement is controlled by a number of vernalization genes designated *VRN1* to *VRN5* (McIntosh et al. 2003). The *Triticum* vernalization genes *VRN1* and *VRN2* have been mapped to the middle of the long arm of homoeologous group 5 and on the distal region of the long arm of homoeologous group 4, respectively (Dubcovsky et al. 1998). Their chromosome locations suggest that *VRN1* and *VRN2* are not related to the *VIL* genes, a fact that was confirmed by the recent cloning of the two vernalization genes (Yan et al. 2003, 2004a). The *VRN-B4* gene, recently re-designated *VRN-B3* (Yan et al. 2006), has been mapped on the short arm of chromosome 7B (Law and Wolfe 1966), which indicates that allelic variation at this locus is not related to the *VIL* loci.

TmVIL1 was mapped completely linked to centromeric markers on chromosome 5A^m, a location that is close but not identical to the one reported for the vernalization gene *VRN-D5* from 'Triple Dirk F' (Kato et al. 2003). *VRN-D5* was mapped 4.4 cM distal to

microsatellite marker *Xgdm3* (Kato et al. 2003), which is located on the 5D long arm 6–12 cM distal to the centromere (Pestsova et al. 2000). This places *VRN-D5* 10–16 cM distal to the centromere and not exactly in the *TmVIL1* region (Fig. 4).

TmVIL2 was mapped on the centromeric region of homoeologous group 6, which does not correspond to any of the mapped vernalization loci in wheat and barley. However, in Chinese Spring, increased dosage of group 6 chromosomes delays ear-emergence whereas reduced dosage of the same chromosomes accelerates ear emergence. These effects are removed by vernalization (Islam-Faridi et al. 1996). To see if there is a relationship between the gene controlling these differences in flowering time and *TmVIL2*, it would be necessary to map the flowering gene on chromosome 6 more precisely.

TmVIL3 was mapped to the centromeric region of chromosome 1, which coincides with the original location reported for the *VRN-H3* vernalization gene from barley. This preliminary result was based on the loose linkage of *VRN-H3* with a morphological marker (45 cM) located on chromosome 1H (Yasuda 1969). However, more precise mapping results have confirmed that *VRN-H3* is actually located on homoeologous group 7 (Yan et al. 2006), eliminating *TmVIL3* as a candidate for *VRN3*.

Conservation of *VIL* gene structure

The Arabidopsis and the grass *VIL* proteins share several conserved structural elements. Proteins from both

species have a conserved PHD finger motif, a zinc-finger motif characterized by a conserved Cys₄-His-Cys₃ (C4HC3) pattern (Aasland et al. 1995). PHD fingers are found in a large number of genes associated with chromatin-mediated transcriptional regulation (Aasland et al. 1995; Sung and Amasino 2004a). The VIN3 and VIL1 mediated chromatin modification is essential to the establishment of a mitotically stable vernalized state and the epigenetic control of vernalization in Arabidopsis (Sheldon et al. 2002; Amasino 2004; Sung et al. 2006).

The *TmVIL* proteins also have a putative FNIII domain as reported before for the *AtVIN3/AtVIL* genes (Sung et al. 2006). The FNIII domain is one of the repeated structural motifs discovered in Fibronectin (FN) (Pankov and Yamada 2002) and found to be involved in protein–protein interactions (Aspberg et al. 1997). The specific role of this domain in the *VIN3/VIL* genes is currently unknown.

Finally, the wheat *VIL* proteins carry a carboxy terminal VID domain, which is conserved among all protein members of this family. Two-hybrid assays using a series of *AtVIN3* deletions and other *AtVIL* proteins demonstrated that the VID domain is necessary and sufficient for positive two-hybrid interactions (Sung et al. 2006).

Transcription profile of *TmVIL* genes

One of the most interesting results from this study was the up-regulation of the wheat *VIL* genes by vernalization and SD. Although a basal transcript level of the three *VIL* genes was observed in the leaves of wheat plants grown under warm conditions, a significant increase of the *VIL* transcript levels occurred after long-term exposure to cold conditions, during both SDs and LDs. Depending on the gene, an increase in transcript levels continued up to the fourth or sixth week of vernalization (Fig. 5). Another similarity between the transcription profiles of the three *TmVIL* genes and *AtVIN3* was the transient effect of the vernalization up-regulation. When plants were returned to warm conditions after vernalization, transcript from all three *TmVIL* genes reverted to their pre-vernalization levels (Fig. 5).

The *TmVIL* genes showed relatively high-basal transcript levels (only 6–21-fold lower than the highly expressed ACTIN endogenous control) that transiently increased during vernalization. Compared to the Arabidopsis genes, this transcription profile is more similar to *AtVIL1* and *AtVIL2* than to *AtVIN3*. *AtVIN3* transcript levels are almost undetectable before vernalization or within 3 days after plants were

returned to a warm growth temperature after vernalization (Sung and Amasino 2004a).

In addition to responding to vernalization, Arabidopsis *VIL1* is also responsive to photoperiod (Sung et al. 2006). SD promotes the transcription of Arabidopsis *VIL1* and early flowering under non-vernalized SD conditions. Transcript levels of the wheat *VIL* genes were generally higher under SD conditions, but these differences were detected only during the early developmental stages for the *TmVIL1* and *TmVIL2* genes and later in development for the *TmVIL3* gene.

We currently do not know if the higher transcript levels of the *TmVIL* genes observed under vernalization and SD are the result of transcription up-regulation or increased stability of the corresponding mRNAs.

Biological role of *VIL* genes

In Arabidopsis, the cellular memory of the vernalized state is mediated by histone modifications of regulatory regions located at the promoter and the first intron of *FLC*, a central flowering repressor down-regulated by vernalization (Michaels and Amasino 1999; Bastow et al. 2004; Sung and Amasino 2004a, b). The establishment of a silenced *FLC* state requires histone H3 deacetylation of Lys 9 and Lys 14 and dimethylation of Lys 9 and Lys 27 (Bastow et al. 2004; Sung and Amasino 2004a). Presumably, vernalization-induced expression of *AtVIN3* is required for the H3 deacetylation in *FLC* chromatin, which in turn promotes the chromatin dimethylation at H3 Lys 9 and Lys 27 (Sung and Amasino 2004a).

Plant homeodomain finger containing proteins such as *AtVIN3* are often members of multi-subunit chromatin-remodeling complexes. This was recently confirmed by studying other *AtVIN3* like genes in Arabidopsis. The *AtVIN3* protein was shown to interact with the *AtVIL1* and *AtVIL2* proteins in yeast-two-hybrid experiments (Sung et al. 2006). A recent study also suggests that *AtVIN3* and *AtVRN2* are part of a large protein complex ($\approx 1,000$ kDa) containing a number of polycomb-group (PcG) proteins, which are required for the repression of *FLC* by vernalization (Wood et al. 2006).

The characterization of *vin3* and *vil1* mutants confirmed that both genes are required for the vernalization-mediated enrichment of di- and tri-methylation at the H3 Lys 9 and Lys 27 of *FLC* chromatin. Similarly, both alleles are needed for chromatin changes in *FLM*, an *FLC*-related flowering repressor. The *FLC*-independent vernalization pathway (Michaels and Amasino 2001) also requires a functional *AtVIN3* allele

(Sung and Amasino 2004a). One of the genes from this pathway, the MADS-box gene *AGL19*, is a flowering promoter normally repressed by several PcG proteins. Vernalization relieves *AGL19* from the PcG repression by a mechanism that requires a functional *AtVIN3* allele (Schonrock et al. 2006). These results suggest the possibility that the vernalization and photoperiod responses of the wheat *VIL* genes might be related to the regulation of other MADS-box gene(s) different from *FLC*, which has not been detected so far in the temperate cereals.

Arabidopsis vill mutants exhibit a substantial delay of flowering under SD independent of vernalization, suggesting that *AtVIL1* promotes flowering under SD. Sung et al. (2006) hypothesized that the *AtVIL1* mediated repression of *FLM* under SD serves to attenuate the photoperiodic response.

The similar effects of SD and vernalization on the transcript levels of *Arabidopsis* and wheat *VIL* genes, together with their conserved structural features, suggest that this group of genes might have related functions. It would be interesting to produce wheat *VIL* mutants to test their effect on flowering time under SD conditions, vernalization requirement, and chromatin remodeling of genes involved in the regulation of flowering in the temperate cereals. We have initiated a wheat TILLING project to generate *VIL* mutants and further explore their role in the regulation of flowering in temperate cereals.

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