A PHD finger protein involved in both the vernalization and photoperiod pathways in *Arabidopsis*

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The proper timing of flowering is critical for successful reproduction. The perception of the seasonal cues of day-length changes and exposure to cold influences flowering time in many plant species through the photoperiod and vernalization pathways, respectively. Here we show that a plant homeodomain (PHD) finger-containing protein, VIN3-LIKE 1 (VIL1), participates in both the photoperiod and vernalization pathways in *Arabidopsis thaliana* by regulating expression of the related floral repressors *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS M* (*FLM*). In the vernalization pathway, VIL1, along with VERNALIZATION INSENSITIVE 3 (VIN3), is necessary for the modifications to *FLC* and *FLM* chromatin that are associated with an epigenetically silenced state and with acquisition of competence to flower. In addition, *VIL1* regulates *FLM* independently of *VIN3* in a photoperiod-dependent manner.

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The transition to flowering is a critical developmental event in the plant life cycle. In many plants, the perception of seasonal changes affects the timing of this transition. Two environmental cues that many plant species monitor for flowering time control are the prolonged cold of winter and changing day length; these cues promote flowering through the vernalization and photoperiod pathways, respectively. Most accessions of *Arabidopsis thaliana* exhibit a facultative response to these signals: In the absence of either environmental cue, flowering is delayed but nevertheless eventually occurs.

In *Arabidopsis*, vernalization results in the mitotically stable repression of the potent floral repressor, *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino 1999; Sheldon et al. 1999). Vernalization-mediated repression of *FLC* is associated with histone modifications such as methylation of histone H3 Lys 9 (H3K9) and Lys 27 (H3K27) as well as H3 deacetylation (Bastow et al. 2004; Sung and Amasino 2004). A plant homeodomain (PHD) finger-containing protein, *VERNALIZATION INSENSITIVE 3* (*VIN3*), is required for *FLC* repression by vernalization and the associated modifications to *FLC* chromatin (Sung and Amasino 2004). Here we show that a related PHD finger-containing protein, VIN3-LIKE 1 (VIL1), identified in a screen for proteins that interact with VIN3, cooperates with VIN3 in the vernalization-mediated repression of *FLC*. Furthermore, independently of *VIN3* activity, *VIL1* mediates the photoperiod-specific repression of another member of the *FLC* clade. Thus, *VIL1* is involved in the regulation of flowering by two environmental-sensing pathways.

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Results and Discussion

Because VIN3 is essential for the vernalization response (Sung and Amasino 2004) and PHD finger-containing proteins are often members of multisubunit chromatin remodeling complexes (Bienz 2006), we searched for potential components of a VIN3 complex using the yeast two-hybrid system. This screen revealed two independent VIN3-interacting clones encoding C-terminal regions of At3g24440. Interestingly, At3g24440 encodes a protein similar to VIN3, and thus we designated At3g24440 as *VIL1*. *VIN3* and *VIL1* are members of an *Arabidopsis* clade that encodes proteins sharing sequence and domain structure similarities (Fig. 1A; Supplementary Fig. S1). All members of the VIN3 clade have a conserved C-terminal domain, which to date is unique to the plant kingdom.

That the screen identified clones containing only the conserved C-terminal domain of VIL1 indicated that this region is sufficient to mediate the interaction with VIN3. To delineate the regions of VIN3 that interact with the C-terminal domain of VIL1, we performed two-hybrid assays using a series of VIN3 deletions. This analysis revealed that the conserved C-terminal domain of VIN3 is necessary and sufficient for the two-hybrid interaction (Fig. 1B). We refer to this C-terminal domain as the VIN3-Interacting Domain (VID). Furthermore, pairwise examination of the interactions among the VID regions of VIN3 and other family members indicated that, in addition to the VIN3:VIL1 interaction, VIL2 interacts strongly with itself and weakly with VIN3 and VIL1 (Fig. 1C).

To evaluate whether *VIL1* plays a role in vernalization, we isolated two T-DNA insertion mutants (SALK_136506, *vil1-1*; SALK_140132, *vil1-2*) and introduced these *vil1* alleles into the vernalization-requiring genetic background *FRI*-Col (the Columbia accession into which *FRIGIDA* has been introgressed as described in Lee and Amasino [1995]). *VIL1* is indeed required for vernalization: *vil1* mutants exhibit an impaired vernalization response similar to that of *vin3* (Fig. 1D). Furthermore, lesions in *vin3* and *vil1* also cause the similar molecular phenotypes of incomplete repression of *FLC* during cold exposure and a lack of *FLC* repression after cold exposure (Fig. 1E).

A molecular phenotype of vernalization is an enrichment of H3K9 and H3K27 dimethylation at *FLC* chromatin (Bastow et al. 2004; Sung and Amasino 2004). However, for the following reasons, we evaluated whether H3K9 and H3K27 trimethylation of *FLC* chro-

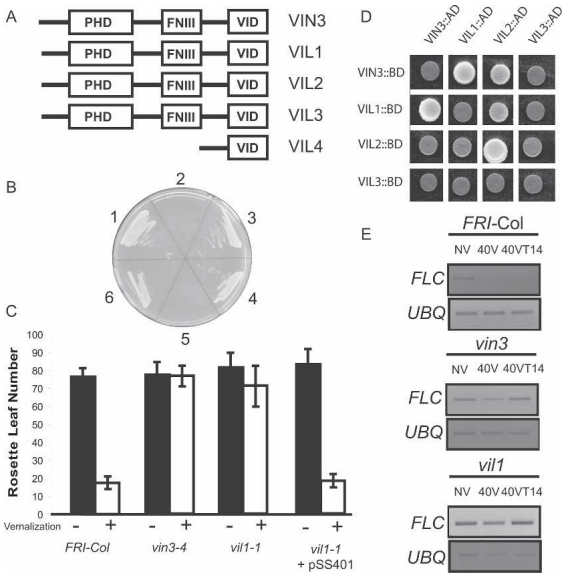

Figure 1. (*A*) Domain structures of VIN3-like proteins. (Amino acid alignments are provided in Supplementary Fig. 1.). VIN3 is At5g57380; VIL1 is At3g24440; VIL2 is At4g30200; VIL3 is At2g18880; and VIL4 is At2g18870. (*B*) The VID region is required for the interaction between VIN3 and VIL1. The constructs either have the VID region alone as described in Supplementary Figure S1 or the remainder of the gene without the VID region. Yeast two-hybrid assays: (*1*) full-length VIN3 as bait and full-length VIL1 as a prey. (*2*) VIN3 without VID as bait and full-length VIL1 as a prey. (*3*) VID of VIN3 as bait and full-length VIL1 as prey. (*4*) Full-length VIL1 as bait and full-length VIN3 as prey. (*5*) Full-length VIL1 as bait and VIN3 without VID as prey. (*6*) Full-length VIL1 as bait and VID of VIN3 as prey. Yeast two-hybrid assay conditions are described in Materials and Methods (Supplemental Material). (*C*) Yeast two-hybrid assays among the VID regions. (*D*) Vernalization response in *vil1* and *vin3*. (Filled bars) Leaf number at flowering of nonvernalized plants; (open bars) values for plants cold-treated for 40 d as described in Materials and Methods (Supplemental Material). (*E*) *FLC* mRNA levels in *vin3* and *vil1*. (NV) Samples from nonvernalized plants grown at 22°C; (40V) samples prepared directly from plants grown for 40 d at 4°C; (40VT14) plants grown for 40 d at 4°C followed by growth at 22°C for 14 d.

matin might also be associated with the vernalized, repressed state. First, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), the plant homolog of HETEROCHROMATIN PROTEIN 1 (HP1), is associated with *FLC* chromatin after vernalization (Sung et al. 2006). On a genome-wide level, LHP1 localizes predominantly to regions of euchromatin (Libault et al. 2005; Nakahigashi et al. 2005; Sung et al. 2006), and a recent study indicates that in plants, trimethylated H3K27 also localizes to regions of euchromatin (Mathieu et al. 2005). Second, trimethylated H3K9 is more specifically associated with repressed genes that are bound by HP1 than is dimethylated H3K9 (Jacobs and Khorasanizadeh 2002). Vernalization results in an enrichment of both H3K9 and H3K27 trimethylation at *FLC* chromatin in a pattern similar to the changes in dimethylation (Fig. 2B–E). Notably, regions at which trimethylation of H3K9 and H3K27 is enriched in *FLC* chromatin (Fig. 2D,E; Supplementary Fig. S2) are correlated with regions at which LHP1 is also enriched by vernalization (Sung et al. 2006). Thus, although at a genome-wide level trimethylation of H3K9 and H3K27 exists at low levels in *Arabidopsis* (Johnson et al. 2004), these modifications may play a key role in the repression of developmentally regulated genes in *Arabidopsis*.

In a *vil1* mutant, the histone methylations characteristic of the vernalized state of *FLC* chromatin do not occur (Fig. 2). Thus, that the physical interaction between VIN3 and VIL1 observed in a two-hybrid assay is critical for vernalization is consistent with the fact that both *vin3* and *vil1* mutants exhibit similar phenotypes including an impaired flowering response to a vernalizing cold treatment as well as an inability to initiate the modifications to *FLC* chromatin associated with vernalization.

VIN3 is only expressed in response to long periods of cold exposure (Fig. 3A; Sung and Amasino 2004); however, cold exposure has little effect on *VIL1* mRNA levels. In short days (SD; 8 h light/16 h dark), *VIL1* is highly expressed before, during, and after cold exposure with perhaps only a slight increase in mRNA levels during cold exposure (SD are standard conditions to evaluate the effect of cold exposure on mRNA levels because vernalization typically occurs during the SD of winter) (Fig. 3A). The temperature-independent expression of *VIL1* indicates it may have other roles in flowering in addition to its role in vernalization. Therefore, we evaluated the flowering behavior of *vil1* mutants in a variety of conditions. A *vil1* lesion does not affect the flowering behavior of nonvernalized *FRI*-Col grown in inductive long days (LD; 16 h light/8 h dark) (Fig. 1D), nor does it affect flowering in LD in the wild-type Col background (i.e., Col that does not have an active *FRI* allele) (Johanson et al. 2000). However, *vil1* mutants exhibit a substantial delay of flowering in SD (Fig. 3B; data shown for Col lines without an active *FRI* allele). Thus, in addition to being required for vernalization, *VIL1* promotes flowering in SD. This is in contrast to the vernalization-spe-

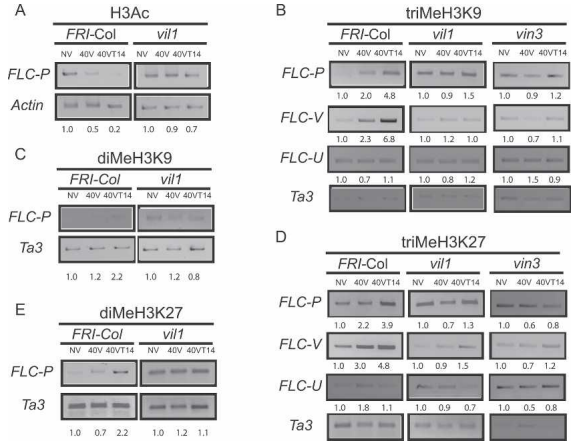

Figure 2. Chromatin immunoprecipitation (ChIP) analyses of vernalization-mediated histone modifications in wild type and *vil1*. Samples are from plants grown as follows: nonvernalized (NV); vernalized for 40 d (40V); vernalized for 40 d and subsequently grown for 14 d at 22°C (40VT14). Relative fold changes are indicated at the *bottom* of each ChIP assay. All assays were repeated with at least two independent chromatin preparations and three independent immunoprecipitations as described in Materials and Methods. Representative ChIP results are shown using antibodies recognizing acetylated Histone H3 (*A*), dimethylated Histone H3 Lys 9 (*B*), dimethylated Histone H3 Lys 27 (*C*), trimethylated Histone H3 Lys 9 (*D*), and trimethylated Histone H3 Lys 27 (*E*). The regions of *FLC* and *FLM* chromatin examined are described in Supplementary Figure S2.

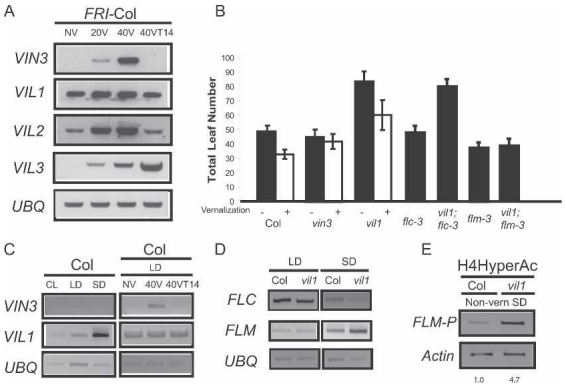

Figure 3. (*A*) Effect of cold exposure on mRNA levels of *VIN3* family members. (*B*) SD flowering behavior. (Filled bars) Leaf number at flowering of nonvernalized plants; (open bars) leaf number of plants vernalized for 40 d as described in Materials and Methods (Supplemental Material). (*C*, *left*) mRNA levels of *VIN3* and *VIL1* in different photoperiod conditions. (CL) Continuous light; (LD) long days, 16 h light/8 h dark; (SD) short days, 8 h light/16 h dark. (*Right*) mRNA levels of *VIN3* and *VIL1* during vernalization under LD. Samples were nonvernalized (NV), vernalized for 40 d (40V), and vernalized for 40 d and subsequently grown for 14 d at 22°C (40VT14). (*D*) mRNA levels of *FLC* and *FLM* in wild-type Columbia (Col) and *vil1* mutants grown in LD and SD. (The stronger *FLC* signal in LD is a result of intentional dilution of the cDNA templates from SD samples relative to LD samples to facilitate evaluation of the increase in *FLM* in SD-grown *vil1* mutants.) (*E*) ChIP assay using antibodies recognizing hyperacetylated Histone H4. Chromatin was prepared from wild-type (Col) and *vil1* mutants grown in SD without vernalization treatment. Three independent immunoprecipitation were performed, and a representative result is shown. Relative fold changes are indicated at the *bottom* of each ChIP assay.

cific role of *VIN3*; *vin3* lesions do not alter the timing of flowering in either LD or SD in nonvernalized plants (Figs. 1D, 3B; Sung and Amasino 2004).

Because *VIL1* promotes flowering in SD, we examined whether *VIL1* expression was photoperiod regulated. *VIL1* is expressed at higher levels in SD than in LD (Fig. 3C), consistent with its role in promoting flowering in SD. Because *VIL1* expression is lower in LD, we also evaluated whether cold exposure might induce *VIL1* in LD, but *VIL1* is not induced during vernalization in these conditions (Fig. 3C). Thus, unlike *VIN3*, which is regulated by cold exposure regardless of photoperiod, the expression of *VIL1* is photoperiod regulated, but not cold regulated (Fig. 3A,C).

Despite the different environmental signals that affect *VIN3* and *VIL1* expression, both *VIN3* and *VIL1* are required for the vernalization-mediated silencing of *FLC* (Fig. 2B). We therefore investigated whether the delayed flowering of a nonvernalized *vil1* mutant in SD is due to altered *FLC* expression. The levels of *FLC* expression in wild type and *vil1* are the same regardless of photoperiod (Fig. 3D; as noted in the legend, the SD samples were diluted relative to LD samples to emphasize the *FLM* mRNA difference). Furthermore, loss of *FLC* does not suppress the late-flowering phenotype of *vil1* mutants in SD (Fig. 3B).

FLC belongs to a clade of genes in *Arabidopsis* (other members: *FLOWERING LOCUS M* [*FLM*]/*MADS AFFECTING FLOWERING 1* [*MAF1*], *MAF2*, *MAF3*, *MAF4*, and *MAF5*) in which certain members, particularly *FLM*, repress flowering (Ratcliffe et al. 2001; Scortecci et al. 2001). Altered *FLM* expression appears to be responsible for the *vil1* SD phenotype. Among the members of the *FLC* clade, only *FLM* is up-regulated in *vil1* mutants in SD compared with the wild type (Fig. 3D; data shown for *FLM*), but in LD, conditions in which loss of *vil1* does not affect the flowering of nonvernalized plants, there is no effect of the *vil1* lesion on *FLM* levels (Fig. 3D). Moreover, loss of *FLM* activity eliminates the late-flowering phenotype of the *vil1* lesion in SD (Fig. 3B). Consistent with the enhanced *FLM* expression in a *vil1* mutant, *FLM* chromatin is more highly acetylated in *vil1* (Fig. 3E). Thus, *VIL1*, in addition to its role in vernalization, functions in the photoperiodic regulation of flowering. Specifically, the *VIL1*-mediated repression of *FLM* in SD serves to attenuate the photoperiod response. In the absence of VIL1 activity, *FLM* mRNA levels are much higher in SD relative to LD, but in wild type, VIL1 attenuates the photoperiod effect on *FLM* expression. The up-regulation of *VIL1* in SD (Fig. 3C) may be necessary for full attenuation of *FLM*.

The additional delay of flowering in a *vil1* mutant in SD is reversed by vernalization (Fig. 3B). At a molecular level, vernalization reduces the elevated expression of *FLM* in *vil1* to the level of nonvernalized wild type, but does not reduce *FLM* expression as completely as that in vernalized wild type (Fig. 4A; note comment on vernalization-mediated *FLM* repression in legend). Thus, as

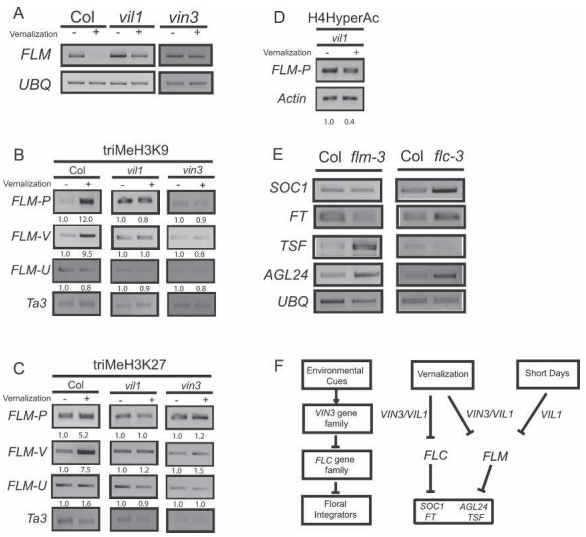

Figure 4. (*A*) Effect of vernalization on mRNA levels of *FLC* and *FLM* in wild-type (Col) and *vil1* mutants grown in SD. Samples in all panels unless noted are nonvernalized (–) or cold-treated for 40 d and subsequently grown for 10 d at 22°C under SD (**+**). (Note: In Scortecci et al. [2001], we reported that *FLM* mRNA levels were not reduced by vernalization in a *FRI*-containing line. However, we now reproducibly detect a vernalization-mediated decrease of *FLM* with or without *FRI*.) (*B*) ChIP assays using antibodies recognizing trimethylated Histone H3 Lys 9 at *FLM* chromatin in wild type (Col), *vin3*, and *vil1*. (*C*) ChIP assay using antibodies recognizing trimethylated Histone H3 Lys 27 at *FLM* chromatin in wild type (Col), *vin3*, and *vil1*. (*D*) ChIP assay using antibodies recognizing hyperacetylated Histone H4 at *FLM* chromatin in *vil1* mutants. (*E*) mRNA expression patterns of floral integrator genes in wild-type (Col), *flm*, and *flc* mutants grown in SD. cDNA templates prepared from mutant seedlings were intentionally diluted relative to wild-type samples to emphasize the increased mRNA levels in mutants. (*F*) Relationship of *VIN3* family genes to the regulatory network controlling flowering time in response to environmental cues.

shown above for *FLC*, *VIL1* is required for the complete vernalization-mediated repression of *FLM*. To investigate the molecular basis of *FLM* repression by vernalization, we examined covalent modifications of *FLM* chromatin during and after cold exposure. Similar to that observed for *FLC* chromatin (Fig. 2B,C), in wild type, vernalization results in a stable increase in H3K9 and H3K27 trimethylation of *FLM* chromatin, but these modifications do not occur in *vil1* or *vin3* mutants (Fig. 4B,C). Thus, both *VIN3* and *VIL1* are required for the vernalization-mediated increase in tri- H3K9 and H3K27 methylation of *FLM* chromatin. As noted above, there is an additional increment of *FLM* expression that occurs in SD in a *vil1* mutant that can be repressed by vernalization without *VIL1* activity (Fig. 4A) and without an apparent increase in the H3K9 and H3K27 trimethylation of *FLM* chromatin (Fig. 4B,C). In a *vil1* mutant background, however, there is a vernalization-mediated decrease in levels of H4 acetylation of *FLM* chromatin (Fig. 4D). Thus, although *VIL1* contributes to *FLM* repression in SD and to the strong silencing of *FLM* by vernalization, there is a *VIL1*-independent mechanism that can reduce the ectopic *FLM* expression of a *vil1* mutant during vernalization.

Although both *FLC* and *FLM* function as floral repressors, *FLC* appears to have a greater role than *FLM* in repressing *SOC1* and *FT* (Fig. 4E), consistent with previous reports that both *SOC1* and *FT* targets are bound directly by *FLC* (Helliwell et al. 2006; Searle et al. 2006). Interestingly, the mRNA levels of *TWIN SISTER OF FT* (*TSF*), a relative of *FT* that also promotes flowering (Michaels et al. 2005; Yamaguchi et al. 2005), are more affected by loss of *FLM* than by loss of *FLC* (Fig. 4E). Thus, there appears to be specialization in the roles of *FLC* and *FLM* in repressing specific floral activators. This specialization, along with the role of *VIL1* but not *VIN3* in the photoperiodic control of flowering, illustrates the intricate regulatory network between the *VIN3* and the *FLC* gene families and downstream "integrators" of flowering pathways (Fig. 4F).

In conclusion, *VIN3* and *VIL1* are required for the vernalization-mediated changes in the chromatin structure of *FLC* and *FLC* relatives like *FLM* that lead to the establishment of competence to flower characteristic of the vernalized state. *VIL1* has the additional role in the photoperiodic regulation of flowering time of attenuating the level of *FLM* expression in noninductive photoperiods. Thus, in *Arabidopsis*, two members of the *VIN3* family have evolved to transduce perception of the environmental cues of a vernalizing cold treatment and SD photoperiods into regulation of target genes. The *VIN3* gene family is found in other plant species, and it will be interesting to evaluate whether *VIN3* family members are involved in the flowering response to environmental signals in other species.

Materials and methods

Plants materials

T-DNA insertion mutants for At3g24440 were obtained from *Arabidopsis* Biological Resource Center (ABRC) (Alonso et al. 2003), and a homozygous line for SALK_136506 was designated as *vil1-1* and SALK_140132 as *vil1-2*. *vil1-1* and *vil1-2* in Columbia were introgressed into *FRI*-Col (Lee and Amasino 1995). A T-DNA insertion mutant for *FLM* (SALK_141971) was also obtained from ABRC (Alonso et al. 2003), and a homozygous line was designated as *flm-3*. *flc-3* was previously described (Michaels and Amasino 1999).

Plant transformation

The coding region of *VIL1* was first subcloned into the pENTR directional TOPO vector (Invitrogen) followed by LR reaction (Invitrogen) into the destination vector pEarleyGate100 (Earley et al. 2006) using LR clonase enzyme (Invitrogen). The resulting construct was designated pSS401 and transformed into *Agrobacterium* and used for plant transformation. Plant transformations were done as described (Clough and Bent 1998).

Yeast two-hybrid assays

cDNAs were amplified using RT–PCR and subcloned into the pENTR directional TOPO vector (Invitrogen) followed by LR reaction (Invitrogen) into destination vector pDEST32 and pDEST22 using LR clonase enzyme (Invitrogen). Yeast transformation and analyses were performed using the ProQuest Two-Hybrid System with Gateway Technology (Invitrogen). Interactions were verified by growing yeasts on plates with SC–Trp–Leu–His + 3AT (100 mM).

Vernalization treatment

Seeds were germinated on agar plates for 5 d at 22°C and vernalized for 40 d at 4°C under 8 h of light and 16 h of dark unless indicated otherwise. Post-vernalization samples continued to grow on agar plates under 8 h of light and 16 h of dark at 22°C. All plants samples were prepared at 10 a.m. (2 h after lights on).

Chromatin immunoprecipitation (ChIP) analysis

Chromatin samples were prepared as described (Gendrel et al. 2005). Immunoprecipitations were performed using a ChIP assay kit (Upstate Biotechnology; no. 17-295) following the manufacturer's suggested protocol modified as previously described (Johnson et al. 2002). Antibodies against trimethyl H3K9, trimethyl H3K27, dimethyl H3K9, dimethyl H3K27, and hyperacetylated H4 were obtained from Upstate Biotechnology. Primers used to detect Ta3, actin, and FLC-P, FLC-V, and FLC-U regions were previously described (Sung et al. 2006). The primers used to detect FLM-P were 5′-TTAAATTAGGGCATAACCCTTATCGGAGA-3′ and 5′-GAGAATCGAAAGTTGTCGAGCTTTGTCGA-3′; to detect FLM-V, 5′-CCACTAGACTACAACCAAATGTTCAATGA-3′ and 5′-TGGAGTCAACATCAAGGAAATGAGAAGGA-3′; and to detect FLM-U 5′-GCGTGAAGAACCCTAGACCTTCTTATTTC-3′ and 5′-AA CATGAAACAGAGTTCAAAAGCTGGCTT-3′. Fold changes were calculated compared with controls using ImageQuant. All ChIP assays were performed at least three times from at least two chromatin samples prepared from biological replicates. It should be noted that unlike ChIP using antibodies against dimethyl H3K9 and dimethyl H3K27, Ta3 sequences were not well amplified from ChIP samples using antibodies against trimethyl H3K9 and trimethyl H3K27, consistent with previous reports (Johnson et al. 2004; Mathieu et al. 2005). Thus Ta3 lanes in these cases only serve as background controls.

RNA analysis

Total RNA was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Isolated RNA samples were further purified using the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed on 2 µg of RNA using the M-MLV System for RT–PCR (Promega) followed by PCR amplification with ExTaq DNA Polymerase (Takara Mirus) according to the manufacturers' recommendations. Primers to amplify *FLC*, FLM, MAF2–5, *UBIQUITIN*, *FT*, *SOC1*, *TSF*, *AGL24*, and *VIN3* were previously described (Ratcliffe et al. 2001, 2003; Michaels et al. 2003; Sung and Amasino 2004). The primer pair used to amplify VIL1 was 5′-CACCTGCAAGAATGCATCGTGTAGAGCTAA TGT-3′ and 5′-TGCCTTTTTAATTGCAGAAGTGCAAAGCT-3′. The primer pair used to amplify VIL2 was 5′-CACCTGCAAAAATTTGGCT TGTCGTGCTGTACT-3′ and 5′-AAGAGATTCAAGAGCAGAAGAG CAAAGTT-3′. The primer pair used to amplify VIL3 was 5′-CACCTGT CAGAACTTGGCTTGCCAGAATAAACT-3′ and 5′-AAGCTCTTTCA AAGCAGAAGAGCAATGTT-3′. Amplified fragments were separated on a 1%–4% agarose gel.

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