A Mechanism Related to the Yeast Transcriptional Regulator Paf1c Is Required for Expression of the Arabidopsis FLC/MAF MADS Box Gene Family

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The Arabidopsis thaliana VERNALIZATION INDEPENDENCE (VIP) gene class has multiple functions in development, including repression of flowering through activation of the MADSbox gene FLC. Epigenetic silencing of FLC plays a substantial role in the promotion of flowering through cold (vernalization). To better understand how VIP genes influence development, we undertook a genetic and molecular study of the previously uncharacterized VIP5 and VIP6 genes. We found that loss of function of these genes also resulted in downregulation of other members of the FLC/MAF gene family, including the photoperiodic pathway regulator MAF1/FLM. We cloned VIP5 and VIP6 through mapping and transcriptional profiling. Both proteins are closely related to distinct components of budding yeast Paf1C, a transcription factor that assists in establishment and maintenance of transcription-promotive chromatin modifications such as ubiquitination of H2B by Bre1/Rad6 and methylation of histone H3 lysine-4 by the trithorax-related histone methylase Set1. Genetic analysis and co-immunoprecipitation experiments suggest that VIP5 and VIP6 function in the same mechanism as the previously described VIP3 and VIP4. Our findings suggest that an evolutionarily conserved transcriptional mechanism plays an essential role in the maintenance of gene expression in higher eukaryotes and has a central function in flowering.

INTRODUCTION

The activity of most eukaryotic genes results from the coordinated effort of a multitude of diverse factors that serve both to recognize the gene and to promote or repress initiation, elongation, and termination of transcription (Lee and Young, 2000). The access of the transcriptional machinery to gene regulatory regions, as well as its progression through transcribed regions, depends both on disruption of higher-order chromatin packaging and the accessibility of DNA at the nucleosomal level (Orphanides and Reinberg, 2000; Svejstrup, 2004). Recent attention in the field of transcription, originating predominately from studies in the budding yeast Saccharomyces cerevisiae, has turned to the astonishing array of factors that modify chromatin structure. These include chromatin-remodeling factors, which displace nucleosomes along the DNA, and histone-modifying enzymes, which add or remove various post-translational modifications including small chemical groups (acetylation, phosphorylation, and methylation) and proteins (ubiquitination and SUMOylation) on nucleosomal histones.

The number and pattern of histone modifications have been hypothesized to play a key role in orchestrating gene activity, both by directly affecting chromatin architecture and by providing interaction sites for other chromatin-associated proteins (Jenuwein and Allis, 2001; Fischle et al., 2003). Superimposed on the complexity of transcription in higher eukaryotes is the requirement to alter gene expression in response to developmental cues and faithfully maintain patterns of gene activity in related cell types in the mature organism. The so-called trithorax group (trxG) and Polycomb group (PcG) proteins have been implicated as having crucial roles in the maintenance of activity states of developmental regulatory genes (Francis and Kingston, 2001). In fruit flies and mammals, trxG and PcG proteins maintain activity or repression, respectively, of the homeotic Hox genes set up during embryogenesis. This activity is accomplished at least in part by the ability of these and associated proteins to carry out and recognize various histone modifications, most notably lysine methylation (Fischle et al., 2003). In plants, although the role of trxG genes has not been well defined, it is becoming increasingly evident that at least the PcG proteins play crucial roles in various developmental progressions through maintenance of the repression of homeotic-function MADSbox genes (Goodrich et al., 1997; Gendall et al., 2001; Kohler et al., 2003).

An excellent model to study the epigenetic dynamics of developmentally important genes in eukaryotes is the silencing
of the Arabidopsis thaliana MADS box floral repressor gene FLC, and associated initiation of flowering, after extended growth of the plant in the cold. Promotion of flowering by long periods of cold, a phenomenon known as vernalization, is an ecologically and agriculturally important response common to many plants and long recognized as having an epigenetic component (Lang, 1965). FLC is one member of a family of six closely related MADSbox proteins in Arabidopsis (Ratcliffe et al., 2001). FLC has been the most extensively studied gene of this family, both because of its substantial effect on the vernalization response and because genetic variation at FLC and its activator FRI are responsible for the natural diversity in flowering habit among Arabidopsis ecotypes (Lee et al., 1993; Johanson et al., 2000; Michaels et al., 2003). The other members of the FLC gene family, designated MAF1-MAF5, can act as floral repressors when expressed constitutively to high levels in transgenic plants, and at least MAF1, MAF2, MAF3, and MAF4 have been shown to be downregulated in vernalized plants (Ratcliffe et al., 2001, 2003). This suggests a conserved function for this clade of MADS box genes in mediating the vernalization response.

Genetic approaches have identified several factors required for silencing of FLC in vernalized plants (Surridge, 2004). These include VRN2, a homolog of the fly PcG protein Su(z)12 (Gendall et al., 2001), VRN1, a putative DNA binding protein (Levy et al., 2002), and VIN3, a plant homeodomain-containing protein (Sung and Amasino, 2004). Examination of vernalization-associated changes in histone modifications of FLC chromatin in wild-type and mutant plants is leading to a framework of a model for the involvement of chromatin changes in FLC silencing (Bastow et al., 2004; Sung and Amasino, 2004). The activity of VIN3, which accumulates during the cold and is associated with deacetylation of histone H3 within FLC promoter and intronic regions, may create favorable conditions for subsequent methylation of H3 at Lys residues K27 and K9 within these regions mediated by VRN2 and VRN1. Although information in plants is limited, studies in animals and fission yeast suggest that methylation at H3K9 within euchromatic regions promotes the formation of heterochromatin and long-term gene silencing, suggesting a precedent for the stable repression of FLC in vernalized plants.

To better understand the dynamics of FLC expression at the molecular level, we have used genetic screens to identify genes required for the maintenance of FLC activity in nonvernalized plants. To date, our group has identified at least 20 loci (Zhang et al., 2003), including seven that comprise the VERNALIZATION INDEPENDENCE (VIP) gene class (Zhang and van Nocker, 2002; Zhang et al., 2003). FLC expression is not detectable in strong vip mutants, indicating a critical function for these genes. VIP3 encodes a protein composed of so-called WD repeats. WD repeat proteins are well represented in eukaryotes, and are believed to coordinate dynamic protein assemblies (van Nocker and Ludwig, 2003). VIP4 encodes a highly charged protein closely related to budding yeast Leo1. Subsequent to our identification of VIP4, Leo1 was identified as a component of a ~1.7 mD transcriptional complex called Paf1C (Mueller and Jaehning, 2002; see below).

Phenotypic analysis of vip mutants suggests that the VIP genes likely have additional roles unrelated to their activation of FLC. For example, vip mutants flower earlier than fcl null mutants, suggesting that other flowering-time genes are targeted (Zhang et al., 2003). In addition, strong vip mutants exhibit mild developmental pleiotropy, which is not seen in an fcl null mutant, suggesting that the VIP genes also target mechanisms unrelated to flowering (Zhang et al., 2003). The objectives of this research were to further characterize the mechanism by which the VIP genes activate FLC, through the identification of the VIP5 and VIP6 genes, and to investigate the role of these VIP genes in FLC-independent flowering and other developmental processes.

RESULTS

VIP5 and VIP6 Function in Concert with VIP3 and VIP4

Based on phenotypic similarity among mutants at the seven VIP loci reported previously, we proposed that the respective genes work in concert in a common mechanism or pathway (Zhang and van Nocker, 2002; Zhang et al., 2003). To explore this idea further, we evaluated the phenotypic effects of combining strong vip3 and vip4 mutations with strong vip5 and vip6 mutations. In short-day photoperiods, where the promotive effects of extended daylengths are minimized, and under a variety of growth temperatures and light intensities, vip5 and vip6 single mutants flowered with a similar number of leaves to either vip3 or vip4 (Figure 1). As previously observed (Zhang et al., 2003), these mutants flowered significantly earlier than an fcl null mutant (Figure 1). There was no significant difference in flowering time between any single mutant and any of the derived double mutant combinations evaluated. In addition, in the double mutants, we did not observe phenotypic effects that were more severe than those exhibited by any single mutant (data not shown). The lack of synergistic effects of coincident inactivity of these genes is consistent with our hypothesis that these genes are closely related in function, possibly as components of a protein complex or molecular pathway.

VIP5 and VIP6 Participate in the Regulation of a Heterogeneous Subset of Genes Including Other Members of the FLC/MAF Gene Family

The observation that strong vip3, vip4, vip5, and vip6 mutants flower earlier than an fcl null mutant suggested that these genes participate in the regulation of flowering-time genes in addition to FLC. The unique (nonredundant) function of the FLC gene appears to be limited to flowering time, because fcl null mutants do not exhibit gross defects beyond timing of flowering. In contrast, the developmental pleiotropy seen in strong vip mutants suggests that these genes participate in the regulation of a subset of genes that include, but are not limited to, FLC (Zhang et al., 2003).

To assist in the identification of these genes, and to evaluate similarity in molecular phenotype between vip5 and vip6 mutants, we performed transcriptional profiling experiments using Affymetrix ATH1 microarrays representing ~22,700 Arabidopsis genes (Figure 2). To eliminate indirect effects on gene expression because of differential activity of FLC and its effects on flowering, we related transcriptional profiles of the strong vip5-1 or vip6-3 mutant plants, in which FLC transcripts are not detectable, with...
Flowering time (measured as the total number of rosette and cauline leaves produced) is indicated for (A) vip3-1, vip5-1, vip6-3, and derived double mutants and (B) vip4-2, vip5-1, vip6-1, and derived double mutants. Plants were grown under noninductive (8 h light/16 h dark) photoperiods. Results from independent experiments are shown; flowering time of the flc null mutant flc-3 in each experiment is shown for comparison. Values represent the mean and standard deviation for at least 20 plants of each genotype.

Figure 1. Flowering Time of vip3, vip4, vip5, and vip6 Single and Double Mutants.

Flowering time (measured as the total number of rosette and cauline leaves produced) is indicated for (A) vip3-1, vip5-1, vip6-3, and derived double mutants and (B) vip4-2, vip5-1, vip6-1, and derived double mutants. Plants were grown under noninductive (8 h light/16 h dark) photoperiods. Results from independent experiments are shown; flowering time of the flc null mutant flc-3 in each experiment is shown for comparison. Values represent the mean and standard deviation for at least 20 plants of each genotype.

The essentially indistinguishable phenotype conferred by strong mutation at each vip locus (Zhang et al., 2003) suggested that a similar subset of genes would be affected in each mutant. This was indeed the case with vip5 and vip6. The data for these two mutants revealed a high degree of overlap (Figure 2); the subset of genes that showed a strong decrease in both mutants represented 79% of the strongly decreased genes in vip5, and 77% of the strongly decreased genes in vip6 (see Supplemental Table 1 online). The degree of overlap was nearly complete when the subset was defined by slightly relaxed criteria for either of the pairwise comparisons (see Methods). For example, all of the 42 genes exhibiting a strong decrease in vip5, as defined by the more stringent criteria, also met the relaxed criteria for a decrease in vip6 (see Supplemental Table 1 online). The overlap was also apparent when the data for vip5 and vip6 were compared directly using the more stringent criteria; for example, only two genes showed a strong decrease in vip6 relative to vip5, and one of these was subsequently identified as the VIP6 gene itself (Figure 2 and data not shown).

Interestingly, among the genes showing decreased expression in both vip5 and vip6 was the FLC paralog MAF1 (Ratcliffe et al., 2001; also known as FLM [Scortecchi et al., 2001]). We confirmed this result through RT-PCR analysis (Figure 3). Like FLC, MAF1 acts as a repressor of flowering, and at least in the Columbia (Col) background is downregulated in vernalized plants (Ratcliffe et al., 2001; our unpublished results). The FLC/FLM MADS box clade in Arabidopsis is represented by four additional genes, designated MAF2-MAF5, that also can act as floral repressors (Ratcliffe et al., 2001, 2003). We considered whether VIP5 and VIP6 also participate in the regulation of these genes. MAF2, MAF4, and MAF5 were also represented on the microarrays, but their expression was statistically undetectable (MAF2 and MAF4) or did not exhibit a significant change (MAF5) in our microarray data. However, RT-PCR analysis indicated a modest but reproducible decrease in MAF2, and a marked silencing of the remaining MAF genes, in both vip5 and vip6 mutants, relative to the flc null (Figure 3). Notably, the involvement of VIP5 or VIP6 in the activation of the MAF genes did not depend on FLC activity, because this experiment was performed in an flc null genetic background. This suggests that the MAF gene family members represent additional regulatory targets of VIP5 and VIP6.

VIP6 Encodes a Plant Homolog of the Pf1C Component Ctr9

The VIP6 gene was represented by three alleles derived from fast-neutron mutagenesis (vip6-1) and T-DNA mutagenesis (vip6-2 and vip6-3) that, based on phenotypic similarity of the respective mutants, were of equivalent severity (data not shown). Initial attempts to identify VIP6 by characterizing genomic DNA flanking the T-DNA insertion site in the vip6-2 or vip6-3 mutants were not successful. Therefore, we used a positional cloning approach, and localized VIP6 within a ~1.2-mb region of chromosome II (Figure 4A). Because no recombination was detected in the immediate region of VIP6, we analyzed the activity of the majority of genes within the ~1.2-mb region in the vip6-3 mutant using data derived from microarray hybridizations (above). A single
analyzed gene within this region, designated At2g06210, showed a statistically significant decreased expression in the vip6-3 mutant as compared with the flc-3 mutant (Figures 2 and 4B; data not shown). We were not able to detect At2g06210 transcripts in wild-type plants by RNA gel blotting, even using phosphorimaging and extended exposures. However, analysis of the At2g06210 gene by RT-PCR in wild-type plants and in the strong vip6-1 mutant revealed a strong decrease in mRNA accumulation in the mutant (Figure 5A). PCR analysis using T-DNA-specific primers and sets of overlapping primers encompassing the At2g06210 genomic region revealed the presence of T-DNA within the At2g06210 predicted transcribed region in the vip6-3 mutant (Figure 4A and data not shown). We were unable to amplify any region of At2g06210 genomic DNA from vip6-1 mutant plants, suggesting that the At2g06210 gene was deleted, and that vip6-1 represents a true null allele. As further evidence that At2g06210 represents the VIP6 gene, we analyzed the phenotype of two additional At2g06210 T-DNA insertion mutants from a collection developed at the Salk Institute Genomic Analysis Laboratory (SIGnAL; Alonso et al., 2003). For both lines, plants homozygous for the mutations exhibited a pleiotropic phenotype that was essentially indistinguishable from that of the three previously described vip6 mutants. The SIGnAL mutant alleles were isolated in the Col background, which does not strongly express FLC and flowers soon after germination; when introduced into the synthetic, winter-annual Col:FRI{sup S2} background, these alleles conferred early flowering and loss of FLC expression (data not shown). Finally, antibodies raised against a portion of the At2g06210 protein recognized a ~130-kD species in wild-type plants that was absent in plants carrying strong vip6 alleles (Figure 4C). Based on these observations, we concluded that At2g06210 is VIP6.

Transgenic antisense expression of a VIP6 cDNA in a wild-type (Col:FRI{sup S2}) background conferred a broad degree of acceleration of flowering time, with approximately one-half of initial transformants (T1 plants) flowering during the course of the experiment, and the earliest flowering plants (~10% of the population) flowering at approximately the same time as vernalized wild-type plants (see Supplemental Figure 1 online). Interestingly, only a minor fraction of VIP6 antisense plants exhibited developmental pleiotropy. As with flowering time, a range of pleiotropy was seen, with the most severe effects limited to the earliest-flowering T1 individuals. However, several of the earliest-flowering T1 plants did not exhibit obvious phenotypic defects other than flowering timing (data not shown).

We found that VIP6 transcript and protein levels were similar in vernalized and nonvernalized plants (Figure 5), suggesting that vernalization-mediated silencing of FLC does not directly involve modulation of VIP6 expression. Also, VIP6 mRNA and protein were expressed at wild-type levels in the Col genetic background, which lacks a functional FRI allele (Figure 5), suggesting that VIP6 does not regulate FLC downstream from FRI. Immunoblot analysis of dissected whole plants indicated that the VIP6 protein is ubiquitously expressed, with the strongest accumulation in apical tissues (data not shown).

We also detected VIP6 mRNA expression at wild-type levels in strong vip3, vip4, and vip5 mutants (Figure 5A), suggesting that the VIP6 gene was not subject to regulation by these other VIP

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**Figure 2.** Characteristics of Microarray Data Derived from flc, vip5, and vip6 Mutants.

Signal intensity was plotted to compare single replicates of flc with vip5 (top), flc with vip6 (middle), or vip5 with vip6 (bottom). The signal positions for FLC and/or VIP6 are indicated. For each comparison, representative data are shown.
the VIP6 protein was not easily detectable in strong genes. Interestingly, however, in contrast with wild-type plants, the VIP6 protein was not easily detectable in strong vip3 genes, including human, fruit fly, frog, slime mold, rice, and yeasts (see Supplemental Figure 2 online; data not shown). Of these, only the Ctr9 protein from budding yeast has been functionally characterized. This protein has been described as a component of Paf1C (Mueller and Jaehning, 2002), a transcription factor required for specific transcription-promotive covalent modifications of chromatin-associated histones: ubiquitination of H2B within its C-terminal domain, and methylation of H3 at residues K4, K36, and K79 (Ng et al., 2003a; Wood et al., 2003b). Paf1C is associated with the initiating and elongating forms of RNA polymerase II (Pol II; Mueller and Jaehning, 2002) and during elongation may serve as a platform for the association of specific histone methylases with chromatin (Hampsey and Reinberg, 2003). It has been postulated that Paf1C provides a mechanism for the memory of recent gene transcription, potentially by antagonizing the activity of silencing proteins and thus reinforcing the active state of genes (Ng et al., 2003b).

Figure 3. Expression of the FLC-Related MAF Genes in flc, vip5, and vip6 Mutants.

Expression was monitored in flc-3, vip5-1, and vip6-3 plants by RT-PCR as described in Methods. Results shown are representative of two independent biological replicates.

genes. Interestingly, however, in contrast with wild-type plants, the VIP6 protein was not easily detectable in strong vip3, vip4, or vip5 genetic backgrounds (Figure 5B). We also observed this effect on VIP6 protein levels in vip1 and vip2 mutants (data not shown). This observation suggests a posttranslational role for these other VIP genes in maintaining VIP6 protein levels.

Based on sequence analysis of several cDNAs, VIP6 could encode two proteins of 1091 and 740 amino acids that would originate from alternative processing of a common precursor RNA (Figures 4A and 4D; see Supplemental Figure 2 online). These putative proteins differ only in the extent of their C termini, and contain so-called tetratricopeptide repeats (TPRs) throughout much of their length. TPRs are ~34-amino acid domains found in proteins of diverse function, and are generally considered to mediate protein-protein interactions and/or assembly of protein complexes (D’Andrea and Regan, 2003). The larger form of the VIP6 protein contains predicted coiled-coil domains near its C terminus, and the C-terminal ~200 amino acid region is highly enriched in charged amino acids such as Glu, Asp, Arg, and Lys (Figure 4D, see Supplemental Figure 2 online). This C-terminal region also contains four potential nuclear localization motifs (Figure 4D), suggesting compartmentalization in the nucleus. Immunoblot analysis using antibodies directed against the N-terminal region of the VIP6 protein recognized only a single, ~130-kD species in wild-type plant extracts, suggesting that the longer form of the protein is relatively more abundant.

A query of public sequence databases identified known and hypothetical VIP6-related proteins in various divergent eukaryotes, including human, fruit fly, frog, slime mold, rice, and yeasts (see Supplementary Figure 2 online; data not shown). Of these, only the Ctr9 protein from budding yeast has been functionally characterized. This protein has been described as a component of Paf1C (Mueller and Jaehning, 2002), a transcription factor required for specific transcription-promotive covalent modifications of chromatin-associated histones: ubiquitination of H2B within its C-terminal domain, and methylation of H3 at residues K4, K36, and K79 (Ng et al., 2003a; Wood et al., 2003b). Paf1C is associated with the initiating and elongating forms of RNA polymerase II (Pol II; Mueller and Jaehning, 2002) and during elongation may serve as a platform for the association of specific histone methylases with chromatin (Hampsey and Reinberg, 2003). It has been postulated that Paf1C provides a mechanism for the memory of recent gene transcription, potentially by antagonizing the activity of silencing proteins and thus reinforcing the active state of genes (Ng et al., 2003b).

The VIP6 Protein Physically Interacts with VIP3 and VIP4 in Vivo

The observation that VIP6 encodes a Paf1C subunit homolog was especially intriguing in light of the previous identification of VIP4 as homologous to yeast Leo1 (Zhang and van Nocker, 2002). Leo1 copurified from yeast cells with Ctr9 and other Paf1C proteins (Mueller and Jaehning, 2002; Krogan et al., 2002; Squazzo et al., 2002), and so is probably an integral subunit of Paf1C. We performed coimmunoprecipitation experiments to determine if, like their yeast counterparts, VIP6 and VIP4 interact in vivo. Indeed, antisera generated against recombinant VIP4 protein (see Supplemental Figure 3A online) specifically immunoprecipitated a ~130-kD protein from wild-type plant extracts that was strongly immunoreactive with anti-VIP6 antibodies (Figure 6A). This protein was absent from parallel immunoprecipitates using extracts from the strong vip6-1 mutant (Figure 6A). Conversely, anti-VIP6 antibodies immunoprecipitated an anti-VIP4 immunoreactive, ~125-kD protein from wild-type extracts that was absent from immunoprecipitates from the strong vip4-2 mutant (Figure 6B). Only a marginally detectable amount of VIP6-immunoreactive protein was immunoprecipitated from vip4-2 extracts with anti-VIP6 IgGs (Figure 6B), consistent with the previous observation that VIP6 protein accumulation is dependent on functional VIP4.

To determine if the VIP6 and VIP4 proteins also interact with the previously described VIP3 in vivo, we constructed and expressed a FLAG-epitope-tagged copy of the VIP3 protein in vip3-1 mutant plants (see Supplemental Figure 3B online). This epitope-tagged VIP3 protein fully complemented the vip3-1 mutant phenotype, indicating that it is functional (data not shown). Anti-FLAG antibodies specifically immunoprecipitated anti-VIP4- and anti-VIP6-immunoreactive proteins of the molecular masses expected for VIP4 and VIP6 (Figure 6C). Based on these observations, we conclude that VIP4, VIP6, and VIP3 interact in a protein complex in vivo.
Figure 4. Map Position, Structure, and Expression of the VIP6 Gene and Protein.

(A) Region of chromosome II containing the VIP6 gene. Molecular markers used in mapping are shown, with genetic distance (recombinations/chromosomes analyzed) between the vip6 mutation and marker indicated. Relevant BAC clones are shown. In the depiction of the VIP6 transcriptional unit, exons are shown as black (translated region) or gray (untranslated region) boxes, and an alternative exonic region detected in some cDNAs is shown as a gray box. The position of the start codon (ATG) and termination codons (TAG and TGA; the TAG termination codon is within the alternative exonic region) are shown. The positions of the T-DNA insertions in the vip6-3 allele and the SIGnAL alleles 090130 and 065364 are indicated.

(B) Expression of VIP6 and adjacent genes in the flc-3, vip5-1, and vip6-3 mutants. Predicted transcriptional units are indicated by arrows. Expression data were derived as described in Methods (+, detected in both replicates; +/− detected in one replicate; −, not detected; NC, no significant change in expression).

(C) VIP6 protein expression in WT and VIP6 mutants. The C-terminal VIP6 protein is indicated by an asterisk.

(D) VIP6 and p150TSP protein expression levels in WT and mutant backgrounds.
VIP5 Encodes an Additional Paf1C Subunit Homolog

The homology of VIP4 and VIP6 with yeast Paf1C components brought up the possibility that other VIP genes encode plant homologs of additional Paf1C subunits. Besides Ctr9 and Leo1, the Paf1C complex includes at least three other proteins: Rtf1, Pafl, and Cdc73. Rtf1 is represented by a single Arabidopsis homolog, designated At1g61040 (data not shown). This gene is located within the likely genetic interval determined for both VIP2 and VIP5 (Zhang et al., 2003), and therefore we explored the possibility that At1g61040 was one of these genes. We sequenced the At1g61040 gene from the vip5-1 mutant and found a small insertion-deletion mutation that would terminate the reading frame after amino acid 319 of the predicted 643-amino acid protein (Figure 7A; see also Supplemental Figure 4 online). RNA gel blotting indicated a ~2.4-kb species that was present at reduced levels in the vip5-1 mutant relative to wild-type plants, suggesting that this mutation affects mRNA accumulation in addition to protein sequence (Figure 7B). We also found that the SIGnAL T-DNA line 062223, which has an insertion within the open reading frame of At1g61040 (Figure 7A), exhibited a phenotype superficially indistinguishable from that of vip5-1 (data not shown). As further evidence that At1g61040 is VIP5, transgenic introduction of a ~5.5-kb DNA containing the At1g61040 transcriptional unit into the vip5-1 mutant background fully complemented the vip5-1 phenotypes (Figure 7A and data not shown). Antisense expression of At1g61040 in a wild-type background conferred a varying degree of early flowering to a majority of primary (T1) transformants. Similar to the effect seen with VIP6-antisense plants, only a fraction of early-flowering plants exhibited strong developmental pleiotropy as seen in the vip5-1 mutant (see Supplemental Figure 1 online). Ectopic expression of VIP5 in transgenic wild-type plants did not confer obvious phenotypic consequences (data not shown).

We found that VIP5 mRNAs were expressed to similar levels in strong vip3, vip4, and vip6 mutants, and, similar to VIP6, were expressed ubiquitously throughout the plant, with strongest accumulation in apices, and were unchanged in vernalized plants or in the Col background (data not shown). Based on current genomic annotation and sequence analysis of several cDNAs, At1g61040/VIP5 encodes a protein containing coiled-coil regions, four potential nuclear localization signal sequences, and a so-called Plus-3 domain (Figure 7C). The Plus-3 domain (PFam accession number PF03126), so named because of the presence of three conserved positively charged residues, has no recognized function, but is found in several other Arabidopsis and eukaryotic proteins (data not shown). The homologous yeast Rtf1 protein also contains these structural features (Figure 7C).

VIP Genes Are Not Required for Global Methylation of Histone H3

The conservation of the VIP4/Leo1, VIP5/Rtf1, and VIP6/Ctr9 proteins, and the role of the Paf1C complex in histone methylation in yeast, suggested that these proteins may be involved in histone methylation in plants. To address this, we examined global histone methylation profiles in strong vip4, vip5, and vip6 mutants. Chromatin histone-enriched proteins were extracted and analyzed by immunoblotting using antisera specific for histone H3 methylated at K4, K36, or K79. In each case, the antibodies reacted strongly with a single species of the predicted appropriate molecular mass (Figure 8), suggesting that these histone modifications are conserved in plants. However, there was no discernible difference in apparent abundance of modified histones in the vip4, vip5, or vip6 mutants when compared with control (flc-3 or Col) extracts. Similar results were obtained with a strong vip3 mutant (Figure 8). Thus, these VIP proteins do not appear to be essential for these histone modifications in
Arabidopsis, at least when assayed in total plant tissues at the whole-genome level.

**DISCUSSION**

Through the work reported here, we show that proteins related to a transcriptional complex from budding yeast are conserved in higher eukaryotes, and in Arabidopsis play a role in the expression of a diverse subset of genes including members of the FLC/MAF family of flowering-time regulators. Our findings add to the increasing complexity of mechanisms of both epigenetic gene regulation and flowering time.

**The VIP Genes Have a Central Role in Flowering through Activation of the FLC/MAF Gene Family**

Our previous observations that vip mutants flower earlier than flc null mutants suggested that other flowering-time genes in addition to FLC are targeted (Zhang et al., 2003). In accordance with this, here we found that loss of VIP5 or VIP6 function led to downregulation of not only FLC, but also other members of the FLC/MAF MADS-box gene family, all of which have the capacity to act as floral repressors (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001). MADS box genes are commonly involved in regulatory cascades, and we considered the possibility that the downregulation of the MAF genes in vip5 and vip6 was mediated through silencing of FLC. However, these experiments were performed in an flc null genetic background and in strong vip mutants where FLC expression was not detected, suggesting that the regulation of the MAF genes occurred independently of FLC activity. Conversely, we considered the possibility that the observed silencing of FLC in the vip5 and vip6 backgrounds was an indirect result of downregulation of MAF genes. However, Ratcliffe et al. (2001, 2003) formerly demonstrated that FLC mRNA abundance was not affected by enhanced, constitutive expression of MAF1 or MAF2, or by mutation in MAF2, suggesting that FLC is normally not subject to regulation by at least these two genes. Therefore VIP5 and VIP6 likely regulate members of the FLC/MAF gene family independently.

The observed common regulation of distinct members of the FLC/MAF gene family by VIP5 and VIP6 is surprising because genetic and molecular analyses have identified clear differences in regulation and function among at least some of these genes. For example, mutation in FLC abrogated the late flowering conferred by functional FRI alleles and loss of function of autonomous pathway genes such as FVE and FCA (Sanda and Amasino, 1996), whereas having little effect on the photoperiodic response (Michaels and Amasino, 2001). In accordance with this, FLC gene expression was found to be strongly activated by FRI and repressed by the autonomous pathway genes, but relatively insensitive to regulation by genes intimately involved in photoperiodic flowering (Sheldon et al., 1999). By contrast, a strong maf1/flm mutation led to substantial loss of the photoperiodic flowering response and abrogated late flowering conferred by mutations in photoperiodic pathway genes (Scortecci et al., 2001, 2003). Also, MAF1/FLM is apparently not subject to appreciable regulation by FRI or autonomous pathway genes (Ratcliffe et al., 2001; Scortecci et al., 2001). Interestingly,
however, like \textit{FLC}, at least the \textit{MAF1-MAF4} genes have been reported to be downregulated after growth in the cold, albeit to different degrees and with different kinetics (Ratcliffe et al., 2001, 2003; our unpublished results).

The regulation of these \textit{FLC/MAF} genes by both cold and the \textit{VIP} genes might suggest a link between vernalization and \textit{VIP} gene function. One possibility is that vernalization attenuates \textit{VIP} activity, potentially through modifying abundance or activity of one or more \textit{VIP} genes/proteins, thus resulting in \textit{FLC/MAF} gene downregulation and silencing. However, we have not observed vernalization-associated changes in mRNA or protein levels for any of the \textit{VIPs} tested, including \textit{VIP5} or \textit{VIP6}. Also arguing against this possibility is the apparent requirement for \textit{VIP} gene activity in unrelated developmental events in vernalized plants, as evidenced by the fact that the molecular and developmental pleiotropy of the respective \textit{vip} mutants is not observed in vernalized wild-type plants (Zhang et al., 2003). Therefore, it is most likely that vernalization and the \textit{VIP} genes regulate \textit{FLC/MAF} genes through independent mechanisms.

**VIP5 and VIP6 Define Important Pleiotropic Regulators of Development**

Our transcriptional profiling experiments identified \textit{FLC} as one of the genes most severely affected in the \textit{vip} mutants (Figure 2), suggesting a special dependence on \textit{VIP} activity. However, we also observed misregulation of a subset of genes not obviously related to \textit{FLC}, and this was expected given the developmental
The VIP Genes Cooperatively Regulate Gene Expression through a Mechanism Related to the Yeast Transcriptional Regulator Paf1C

The seven defined VIP genes carry out a common function in plant growth and development, based on their indistinguishable developmental pleiotropy (Zhang et al., 2003). Consistent with this, we did not observe enhanced effects on flowering time or development when vip3 or vip4 mutations were combined with vip5 or vip6 mutations. A common function for VIP5 and VIP6 was also reflected by the high degree of overlap among misregulated genes in the respective mutants. Because the VIP6 protein is not effectively expressed in the vip5 mutant background, the limited differences that we observed in transcriptional profiles between the vip5 and vip6 mutants could reflect roles for VIP5 that are independent of VIP6. Alternatively, although the vip5 and vip6 mutants were backcrossed to wild-type plants extensively before analysis, these distinctions could also have resulted from genetic lesions unrelated to the vip mutations that were sustained in the original mutagenesis and are still harbored by either vip5 or vip6.

Our observation that the abundance of VIP6 protein, but not mRNA, is dependent on functional VIP3, VIP4, and VIP5 could be explained by reduced posttranslational stability of VIP6 in the absence of participation in a protein complex, presumably involving VIP3, VIP4, and VIP5. The finding that at least VIP3, VIP4, and VIP6 physically interact in vivo is also consistent with the hypothesis that these proteins comprise a protein complex. We formerly reported that VIP4 is a plant homolog of budding yeast Leo1 (Zhang and van Nocker, 2002). Subsequently, it was revealed that Leo1 is a component of the Paf1C transcriptional complex (Mueller and Jaehning, 2002). Here, we identified VIP5 and VIP6 as homologous to the Paf1C components Rtf1 and Ctr9, respectively. These cumulative findings suggest that the Arabidopsis VIP proteins define a plant counterpart of Paf1C.

Figure 8. Immunoblot Analysis of Histone H3 Methylation in Strong vip3, vip4, vip5, and vip6 Mutants, the flc-3 Null Mutant, and the Col Ecotype.

Histone-enriched extracts were resolved by SDS-PAGE and subjected to immunoblotting using antibodies directed against dimethylated Lys-4 (di-M-K4), trimethylated Lys-4 (tri-M-K4), dimethylated Lys-36 (di-M-K36), or dimethylated Lys-79 (di-M-K79). Histone-enriched extracts from human Hela cells, or total protein extracts from wild-type yeast and a yeast rtf1 deletion strain (Δrtf1) are included as controls. The separate images of Hela cell extract results were taken from the same immunoblot. A portion of a representative SDS-PAGE gel (stained with Coomassie blue) is shown to indicate relative quality and quantity of proteins in each lane (total).

VIP4, and VIP6 physically interact in vivo is also consistent with the finding that at least VIP3, VIP4, and VIP6 as homologous to the Paf1C components Rtf1 and Ctr9, respectively. These cumulative findings suggest that the Arabidopsis VIP proteins define a plant counterpart of Paf1C. Consistent with this, we have determined that the At1g79730 gene, which encodes a protein weakly related to the Paf1 component of the Paf1C complex, is likely VIP2 (M.J. Ek-Ramos and S. van Nocker, unpublished results). The WD-repeat protein VIP3 has obvious homologs in animals but not budding yeast (Zhang et al., 2003). WD-repeat proteins are common constituents of large chromatin-associated complexes (van Nocker and Ludwig, 2003), and it is tempting to speculate that VIP3 represents an elaboration of the Paf1C mechanism not relevant for the relatively simple chromatin of yeast.

Although the core components of yeast Paf1C are conserved in higher eukaryotes, their cellular and organismal role has not been explored. The VIP6/Clr9 protein exhibits homology with murine p150TSP. This protein was previously isolated based on its in vitro affinity for an isolated Src homology (SH2) domain, a conserved, ~100 amino acid, phosphopeptide binding module that has been best characterized as a component of proteins with roles in cellular signaling pathways including signal transducer and activator of transcription and suppressor of cytokine signaling proteins, janus kinases, and other tyrosine kinases (Pawson, 2004). Although these signaling pathways are generally not tightly conserved in plants, it remains a possibility that VIP6 couples transcription with plant-specific signaling pathways. In support of this, in vitro binding of p150TSP protein to SH2 is dependent on phosphorylation of Ser/Thr residues and the highly charged C-terminal region of p150TSP (Malek et al., 1996), features that are conserved in VIP6.

Paf1C plays a central role in transcription in yeast and, although not essential for viability, is required for full expression of a variety of yeast genes (Porter et al., 2002). Paf1C
components assist in the ubiquitination of the C-terminal domain of histone H2B by the ubiquitin conjugating/ligase proteins Rad6/Bre1 (Ng et al., 2003a; Wood et al., 2003b). Ubiquitination of H2B within promoter regions, as well as ensuing deubiquitination by the SAGA histone acetyltransferase-associated Ubp8, is required for efficient activation of many genes in yeast (Henry et al., 2003). At least in yeast, H2B ubiquitination is also a prerequisite for methylation of histone H3 at lysines 4 and 79 by the histone methylases Set1/COMPASS and Dot1, respectively, within open reading frames (Wood et al., 2003b). These histone modifications have most often been associated with actively transcribed genes (Hampey and Reinberg, 2003). At least the Rtf1 subunit of Paf1C is also required for efficient, locus-specific H3K36 methylation by an additional histone methylase, Set2, an activity that is apparently independent of H2B ubiquitination (Ng et al., 2003a). Unlike yeast strains deleted for components of Paf1C, Arabidopsis vip mutants did not exhibit detectable defects in methylation at H3K4, K36, or K79 when assayed on a bulk chromatin and total plant tissue basis. Potentially, such an activity is redundant in plants, or occurs in a tissue-specific or locus-specific manner.

Paf1C subunits associate with the initiating and elongating forms of Pol II (Mueller and Jaehning, 2002), and are bound within 5′ regions and open reading frames of various genes (Krogan et al., 2002; Simic et al., 2003). Given the association of Paf1C with elongating Pol II, the capacity of Paf1C to promote H3K4 methylation, and the observation that trimethylation at H3K4 is uniquely associated with actively transcribed loci, together with the apparent lack of enzymes that could demethylate histones, it has been hypothesized that H3K4 trimethylation comprises a molecular memory of recent gene transcription (Ng et al., 2003b). How this memory mechanism is manifested at the molecular level remains mostly unknown. However, methylation of H3K4 can recruit the Isw1 chromatin-remodeling ATPase (Santos-Rosa et al., 2003), which generates specific chromatin of H3K4 can recruit the Isw1 chromatin-remodeling ATPase molecular level remains mostly unknown. However, methylation a molecular memory of recent gene transcription (Ng et al., 2003a). Unlike yeast strains deleted for components of Paf1C, Arabidopsis vip mutants did not exhibit detectable defects in methylation at H3K4, K36, or K79 when assayed on a bulk chromatin and total plant tissue basis. Potentially, such an activity is redundant in plants, or occurs in a tissue-specific or locus-specific manner.

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The prospect that the homologous plant mechanism also participates in FLC transcription through generating active patterns of histone modification is intriguing. In animals, epigenetic maintenance of homeotic gene activity involving the trxG proteins also involves histone methylation at residues including H3K4. For example, the human trx-related MLL (mixed lineage leukemia) protein carries out H3K4 methylation at Hox loci in vivo (Milne et al., 2002). Similarly, in flies, H3K4 methylation by the epigenetic activator Ash1 is essential to maintain activity of homeotic genes in the developing embryo (Tripoulas et al., 1994; Beisel et al., 2002). Similarly to the observed recruitment of Isw1 by methylated H3K4 in yeast, Ash1 activity involves the recruitment of the trxG chromatin-remodeling ATPase Brahma (Beisel et al., 2002). We hypothesize that the role of the VIP proteins in promoting FLC activity in nonvernalized plants is to provide a transcription-associated platform for the modification of FLC chromatin by a trxG-like mechanism, that would antagonize repression by a VRN2-associated PcG mechanism. This effect could involve the recruitment of chromatin-remodeling factors such as PIE1, an ISWI-family protein formerly shown to be required for full expression of FLC (Noh and Amasino, 2003). The only Arabidopsis trx-like protein to have been characterized to date, ATX1, functions in the activation of homeotic genes, and can methylate a synthetic peptide corresponding to the H3 amino-terminus on K4 in vitro (Alvarez-Venegas et al., 2003). A strong atx1 mutation conferred mildly delayed flowering and floral abnormalities that were seemingly distinct from that of the vip mutants (Alvarez-Venegas et al., 2003), suggesting that the activity of ATX1 is not closely tied to that of the VIP genes. However, the Arabidopsis genome encodes for several additional trx-related proteins that could promote FLC expression (Baumbusch et al., 2001).

A very recent study indicates that Paf1C also has transcriptional roles seemingly distinct from chromatin modification (Mueller et al., 2004). Loss of Rtf1 or Cdc73, which dissociated remaining Paf1C proteins from chromatin, conferred only mild phenotypes relative to those resulting from loss of other Paf1C proteins such as Paf1 or Ctr9. Similarly, loss of Paf1 or Ctr9 affected growth to a greater extent than loss of Bre1 or histone methylation (Wood et al., 2003a; Mueller et al., 2004). Moreover, loss of Paf1 or Rtf1 led to global defects in mRNA polyadenylation, suggesting an important function for Paf1C in posttranscriptional events. The increasingly diverse repertoire of activities attributed to Paf1C and its subunits allows wide latitude for speculation on the means by which the related plant VIP mechanism participates in FLC expression, and provides numerous avenues for further exploration. The identification of additional factors required to maintain FLC expression through genetic and biochemical methods holds exceptional promise and may illuminate many more unanticipated connections between basic transcription and development in higher eukaryotes.

METHODS

Plant and Yeast Material and Manipulations

The late-flowering, winter-annual introgression lines Col:FRI<sup>F2</sup> (used here as the wild type) and Ler:FRI<sup>F2</sup>:FLC<sup>F2</sup> were as described previously (Zhang et al., 2003). The null fcl-3 mutant in the Col:FRI<sup>F2</sup> background was a gift from R. Amasino (University of Wisconsin). Populations derived from introgression line Col:FRI<sup>F2</sup> mutagenized by fast-neutron radiation, ethyl methanesulfonate, or T-DNA insertion were as described previously (Zhang et al., 2003). Mutant lines were backcrossed additional two times before microarray analysis. The vip5-1 and vip6-3 lines were backcrossed to wild-type plants an additional two times before microarray analysis. Sequence-indexed T-DNA-mutagenized lines developed at SIGnAL were obtained from the Arabidopsis Biological Resource Center at The Ohio State University (Columbus, OH). Standard genetic techniques were used in the production of double mutants. For vernalizing cold treatments, seeds were allowed to germinate on sterile media (Zhang and van Nocker, 2002) at 5°C. Double mutants. For vernalizing cold treatments, seeds were allowed to germinate on sterile media (Zhang and van Nocker, 2002) at 5°C. Standard genetic techniques were used in the production of double mutants. For vernalizing cold treatments, seeds were allowed to germinate on sterile media (Zhang and van Nocker, 2002) at 5°C. Standard genetic techniques were used in the production of double mutants. For vernalizing cold treatments, seeds were allowed to germinate on sterile media (Zhang and van Nocker, 2002) at 5°C.
Cloning of VIP6

Positional cloning of the VIP6 gene utilized early-flowering F2 progeny of a single F1 individual derived from a cross between vip6-1 and introgression line Ler/FR632-FLC. Bulked-segregant analysis was performed with 24 F2 individuals and molecular markers described by Lukowitz et al. (2000). Fine mapping was done entirely using molecular markers based on small insertion-deletion polymorphisms as characterized and cataloged by Cereon (Cambridge, MA) (http://www.arabidopsis.org/Cereon/index.jsp) and noted in Figure 4A.

Molecular Techniques

For production of VIP6 antisense plants, a 2.6-kb fragment corresponding to a portion of the transcribed region was amplified from apex cDNA using the primers VIP6Fbarm (5'-AAAGGATCTGATTGTGGACAGCAAT-GATTG-3') and VIP6RBam (5'-AAAGGATCCCCTGTTATGTTAGATGAA-ATA-3') and inserted into vector pPZP201:BAR (Zhang and van Nocker, 2002). For production of VIP5 antisense plants, a 2-kb cDNA corresponding to the entire translated region was amplified from shoot apex-derived cDNA using primers VIP5Fbarm (5'-AAAGGATCTCATGATTGTGGACAGCAAT-GATTG-3') and VIP5RBarm (5'-AAAGGATCTGAGAGATGTCGATTTCAAG-3'), and inserted into pPPZP201:BAR:35S. For production of transgenic plants constitutively expressing VIP5, 35S promoter DNA, into the plant genome using a procedure described by the manufacturer. We used protein extracts from inflorescence apices, because VIP4 and VIP6 are strongly expressed in these tissues; ~500 µg of protein extract, in a volume of 500 µL of extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, containing 1 mM phenylmethylsulfonyl fluoride was incubated with 10 µL of IgGs, and mixed continuously for 2 h. Protein A-agarose beads (15 µL) were then added, and the mixture was incubated a further 1 h. Protein A-agarose beads were collected by centrifugation and washed with 1 mL ice-cold washing buffer (extraction buffer lacking Triton X-100) four times. After the final wash, the beads were resuspended in 30 µL of SDS-PAGE sample buffer. All immunoprecipitation procedures were performed at 4°C.

Immunoblotting was done as described by Harlow and Lane (1988), using polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) blocked with Tween-20 in phosphate-buffered saline, and alkaline-phosphatase-labeled, goat anti-rabbit IgGs (Bio-Rad), or enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), using nitrocellulose membranes (Amersham) blocked with 3% skim milk in phosphate-buffered saline, and peroxidase-conjugated, anti-rabbit IgGs (Amersham).

Sequence Analyses

Motifs in the VIP5 and VIP6 proteins were identified using PFam version 13.0 on a Web server maintained by Washington University in St. Louis (http://pfam.wustl.edu/hmmsearch.shtml) or the PredictNLS server at the Columbia University Bioinformatics Center (http://cubic.bioc.columbia.edu/; Cokol et al., 2002). Other sequence analyses were performed using BLAST on Web servers maintained by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) or The Arabidopsis Information Resource (http://www.arabidopsis.org), and programs of the Genetics Computer Group (Madison, WI).

Microarray Analysis

Each of the two replicates for each genotype was composed of three independently derived samples. Each sample included between 12 and 20 plants. Total aerial tissues were harvested when the first flower was fully opened. Total RNA was isolated using Qiagen RNeasy columns (Qiagen, Valencia, CA). Synthesis of cDNA employed the SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA) and 100 pmol of oligo(dT)24 primer (Proligo, Boulder, CO), following the manufacturers’ instructions. Synthesis of biotinylated cDNA utilized the BioArray high yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). The Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara, CA) was used for hybridization. Hybridization and scanning of microarrays was performed at the Genomics Technology Support Facility at Michigan State University.

Microarray data were analyzed using the statistical algorithms within the Affymetrix Microarray Suite (MAS) 5.0 software. We employed pairwise comparisons of the independent biological replicates to identify genes that exhibited a marked change in transcript abundance according to an arbitrary stringent or relaxed definition. For the stringent definition, the gene must have been detected at a statistically significant level (i.e., called present or marginal by the MAS software) in both replicates of either the experiment (vip5 or vip6) or the baseline (fc). In addition, the
MAS software must have observed a statistically significant change in expression (i.e., called decrease, marginal decrease, increase, or marginal increase) in at least three of the four comparisons, and the mean difference in signal intensity must have been threefold or greater. The number of genes that were detected and exhibited a significant change in expression (i.e., detected at least threefold or greater) was, at most, 80 (0.35% of microarrayed genes). For the relaxed definition, the gene must have been detected at a statistically significant level in either replicate of either the experiment or the baseline, the MAS software must have observed a statistically significant change in expression in at least two of the four comparisons, and the mean change in signal intensity must have been twofold or greater. The number of genes that met this relaxed criteria for any one comparison of replicate sample pairs was, at most, 512 (2.25% of microarrayed genes).

Microarray data discussed here have been deposited with the Gene Expression Omnibus database at the NCBI (http://www.ncbi.nlm.nih.gov/geo/; series no. GSE1516).

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