Genetic Analysis of Early Flowering Mutants in Arabidopsis Defines a Class of Pleiotropic Developmental Regulator Required for Expression of the Flowering-Time Switch Flowering Locus C

Hua Zhang,* Callista Ransom,† Philip Ludwig‡ and Steven van Nocker*,†,‡,1

*Programs in *Genetics, †Plant Breeding and Genetics and ‡Cell and Molecular Biology, Michigan State University, East Lansing, Michigan 48824*

Manuscript received October 19, 2002
Accepted for publication January 24, 2003

ABSTRACT

The Arabidopsis flowering-repressor gene *FLOWERING LOCUS C* (*FLC*) is a developmental switch used to trigger floral induction after extended growth in the cold, a process termed vernalization. In vernalized plants, *FLC* becomes transcriptionally silenced through a process that involves an epigenetic mechanism. We identified recessive mutations designated *vernalization independence* (*vip*) that confer cold-independent flowering and suppression of *FLC*. These mutations also lead to developmental pleiotropy, including specific defects in floral morphology, indicating that the associated genes also have functions unrelated to flowering time. We identified the *VIP3* gene by positional cloning and found that it encodes a protein consisting almost exclusively of repeated Trp-Asp (WD) motifs, suggesting that VIP3 could act as a platform to assemble a protein complex. Constitutive transgenic expression of *VIP3* in vernalized plants is insufficient to activate *FLC*, and thus *VIP3* probably participates in the regulation of *FLC* as one component of a more extensive mechanism. Consistent with this, genetic analyses revealed that the *VIP* loci define a functional gene class including at least six additional members. We suggest that *VIP3* and other members of this gene class could represent a previously unrecognized flowering mechanism.

GENETIC and molecular studies in Arabidopsis have shown that flowering results from the action of several interdependent regulatory mechanisms or pathways, each mediating the effect of separate endogenous or environmental influences (Koornneef *et al.* 1998b; Simpson and Dean 2002). The Arabidopsis FLOWERING LOCUS C (FLC) MADS-domain protein is a key flowering-time regulator that integrates the activity of the so-called autonomous pathway and the *FRIGIDA* (*FRI*) gene, which influence flowering independently of day length, and the "vernalization pathway," which moderates the promotive effects of extended growth in the cold (Simpson and Dean 2002). *FLC* in turn represses flowering through negative regulation of the *SOC1* (*AGL20*) and *FT* genes (Lee *et al.* 2000; Samach *et al.* 2000; Michaels and Amasino 2001). In this manner, *FLC* acts antagonistically with *CONSTANS* (*CO*), which moderates the promotive effects of inductive photoperiods (Hepworth *et al.* 2002).

At least six genes, designated *FY*, *FCA*, *FPA*, *FLD*, *LD*, and *FVE*, have been proposed to participate in the autonomous pathway. These genes collectively act to promote flowering through repression of *FLC* (Michaels and Amasino 1999; Rouse *et al.* 2002). In contrast, the activity of *FRI* represses flowering by positively regulating *FLC* expression (Michaels and Amasino 1999; Sheldon *et al.* 1999). The antagonistic relationship between *FRI* and the autonomous pathway has not been clearly defined, but the observation that *FRI* activates *FLC* and represses flowering even in the presence of autonomous pathway activity (*i.e.*, in wild-type plants that carry a functional *FRI* allele) suggests that *FRI* could function to limit the activity of one or more autonomous pathway components.

The early flowering conferred by loss of *FLC* is apparently completely epistatic to the repressive effects of *FRI* or mutation in at least *FCA*, *FVE*, and *LD*, suggesting that the only function of *FRI* or these autonomous pathway genes, with respect to flowering, is to regulate *FLC* (Michaels and Amasino 2001). In noninductive photoperiods, where the promotive influence of the photoperiodic pathway involving *CO* is minimized, plants lacking only *FLC* activity flower earlier than plants lacking only *FRI* activity. This suggests that *FLC* represses flowering to some extent even in the absence of *FRI* and suggests that *FLC* expression is also promoted through mechanism(s) not involving *FRI* (Michaels and Amasino 2001).

Although *FRI* and the known autonomous pathway genes have now been identified at the molecular level, the nature of the corresponding regulatory mechanisms is undefined. Both transcriptional and post-transcriptional events are likely involved, as at least one autonomous pathway gene, *LD*, encodes a homeodomain-transcription factor-like protein (Aukerman and Amasino 1996), and two others, *FCA* and *FPA*, encode proteins

[1]*Corresponding author:* 390 Plant and Soil Sciences Bldg., East Lansing, MI 48824. E-mail: vannocke@msu.edu

with structural features suggestive of RNA binding (Macknight *et al.* 1997; Schomburg *et al.* 2001). Repression of *FLC* by the autonomous pathway is presumed to occur at least partly at the level of *FLC* transcriptional activity, because *FLC* represses flowering when expressed from the constitutive CaMV 35S promoter, even in a genotype lacking strong *FRI* activity (*i.e.*, where the autonomous pathway is active; Michaels and Amasino 1999).

Vernalization is an epigenetic effect (Wellensiek 1962, 1964) associated with suppression of the *FLC* gene (Michaels and Amasino 1999). Vernalization likely targets *FLC* regulatory mechanisms that do not directly involve *FRI* or the autonomous pathway, because cold promotes flowering even in genotypes carrying combined loss-of-function mutations in autonomous pathway genes and in *FRI* (Koornneef *et al.* 1998b). Vernalization-associated *FLC* repression is mediated by at least two genes, *VRN1* and *VRN2* (Simpson and Dean 2002). *VRN2* may act to initiate or maintain a relatively silent state of *FLC* chromatin, as a *vrn2* mutant shows increased accessibility of the *FLC* locus to DNAse I (Gendall *et al.* 2001). Also, the VRN2 protein resembles the Drosophila Polycomb-group transcriptional regulator Su(z)12, which potentially acts at the level of chromatin structure (Gendall *et al.* 2001). Transgenic overexpression of *VRN1*, which encodes a nuclear-localized DNA-binding protein, results in early flowering that is associated with increased expression of *FT*, but not decreased expression of *FLC* (Levy *et al.* 2002). This suggests both that *VRN1* requires vernalization-specific auxiliary factors to target *FLC* and that *VRN1* may also regulate flowering through an *FLC*-independent mechanism. In addition, *VRN1* overexpression disrupts seemingly unrelated developmental processes, indicating that its role may be wider than that of regulating flowering. Neither *VRN1* nor *VRN2* seems to be regulated in a vernalization-associated manner, also revealing that specificity is derived from relationships with cold-regulated factors (Levy *et al.* 2002; C. Dean, personal communication). Genetic analyses suggest that *VRN1* and *VRN2* could represent members of a larger group of genes with similar function (Chandler *et al.* 1996).

Most genotypes of Arabidopsis commonly used in laboratory studies carry a dysfunctional *fri* allele (Johanson *et al.* 2000) and flower soon after germination. Thus, genetic pathways of floral repression in this reference plant have not been extensively characterized. We previously reported the identification and cloning of the *VERNALIZATION INDEPENDENCE 4* (*VIP4*) gene, which acts as a flowering repressor by promoting expression of *FLC* (Zhang and van Nocker 2002). We report here the cloning and characterization of a novel positive regulator of *FLC* designated *VIP3*. On the basis of genetic and molecular evidence presented here, we propose that *VIP3* and, possibly, *VIP4* as well could promote *FLC* expression through a previously unrecognized mechanism and that these genes are members of a functionally similar gene class in Arabidopsis.

MATERIALS AND METHODS

Growth conditions: Arabidopsis seeds were either planted directly into artificial soil mix or surface sterilized and germinated under sterile conditions as described previously (van Nocker *et al.* 2000). Standard growth conditions were 60–100 μmol m^{-2} sec^{-1} of fluorescent lighting in a 16 hr light/8 hr dark photoperiod at 22° and ~50% relative humidity. Short-day growth conditions were identical with standard growth conditions but utilized 8 hr light/16 hr dark photoperiods. For a vernalizing cold treatment, seeds on germination medium were first placed at 4° under 20–50 μmol m^{-2} sec^{-1} of fluorescent lighting in an 8 hr light/16 hr dark photoperiod for 30 or 70 days. For growth under far-red light-enriched conditions, lighting was supplied entirely by household incandescent bulbs.

Strains and genetic techniques: Introgression line Col:*FRI*^{SF2} consists of the dominant *FRI* locus from ecotype San Feliu-2 (*FRI*^{SF2}) introgressed into the Columbia (Col) ecotype (Lee *et al.* 1994). Introgression line L*er*:*FRI*^{SF2}:*FLC*^{SF2} consists of *FRI*^{SF2} and the semidominant *FLC* locus from ecotype San Feliu-2 (*FLC*^{SF2}) both introgressed into the Landsberg *erecta* (L*er*) genetic background through seven successive backcrosses and made homozygous through self-pollination (Lee *et al.* 1994). The *ld-1* mutant (ecotype Col-1) was obtained from the *Arabidopsis* Biological Resource Center (ABRC) at The Ohio State University (Columbus, Ohio). The null *flc-3* mutant was a generous gift from S. Michaels and R. Amasino (University of Wisconsin). The L*er*::*vip3* introgression line was created by carrying out four successive outcrosses to wild-type L*er* and selecting for plants that carried L*er* alleles of *FRI* and *FLC* after the second outcross. The *Escherichia coli* strain harboring bacterial artificial chromosome (BAC) F27B13 was obtained from the ABRC.

Polymerase chain reaction (PCR)-based molecular markers were utilized to discriminate between wild-type and mutant alleles of *VIP3*, *FRI*, and *LD*. A marker for presence of the wild-type *VIP3* allele was designed to amplify, from the wild-type allele, a region spanning the site corresponding to the *vip3* mutation [primers: F27B13.7F2 (5′-TTGCAGGTGGAA GTAGTGCCTC-3′) and F27B13.7 R2 (5′-TGTCATCAGAGA CACTAGCAAGTCG-3′)]. To determine presence of the *vip3* allele, a marker was designed to amplify the right junction of the genomic insertion [primers: F27B13.7F2 and T16L4F (5′-GCCACTGCCGCCAGTTTTATCAAG-3′)]. A marker for discrimination between the *FRI*^{SF2} and *fri*^{Col} alleles was based on a 16-bp length polymorphism within the *FRI* promoter as described by Johanson *et al.* (2000) and employed primers FRI16F (5′-TGGTGTTCCTTCAAACTTTAGG-3′) and FRI16R (5′-GCTCAATCAGTCATTGCACTC-3′). A marker for discrimination between the *LD* and *ld-1* alleles was based on the *ld-1* mutation, which is localized within the *LD* transcribed region, and was generously provided by S. Michaels (University of Wisconsin).

A *vip3/fri* double mutant was created by crossing *vip3* with wild-type Col (carrying the strong, loss-of-function *fri*^{Col} allele). A *VIP3/vip3*, *fri*^{Col}/*fri*^{Col} plant was identified in the respective F_2 population and allowed to self-pollinate, and double mutants were analyzed in the corresponding progeny. A *vip3/fri/ld* triple mutant was created by crossing *vip3* with a plant carrying the strong *ld-1* allele in the Col background. F_2 progeny from this cross that were *fri*^{Col}/*fri*^{Col}, *VIP3/vip3*, and *LD/ld* were allowed to self-pollinate, and triple mutants were analyzed in the corresponding progeny. A *vip3/vip4* double mutant was

created by crossing *vip3* with *vip4-1* and backcrossing the corresponding F_1 plant with a *vip3* mutant. A *vip3/vip3 VIP4-1/vip4-1* plant was allowed to self-pollinate, and double mutants were analyzed in the corresponding progeny.

Mutagenesis and cloning of *VIP3*: For mutagenesis of introgression line Col:FRI^{SF2}, seeds were exposed to ~165 Gy of fast-neutron radiation using the fast-neutron beam at the Michigan State University Cyclotron Laboratory or incubated with 0.15% ethyl methanesulfonate (EMS) overnight and subsequently rinsed extensively with distilled water. Seeds were then subjected to a vernalizing cold treatment and planted in soil, and plants were allowed to self-pollinate. M_2 seed was collected in pools each representing ~1000 M_1 individuals. Approximately 5000 plants from each of 24 fast neutron-derived M_2 families and 20 EMS-derived M_2 families were screened. T-DNA mutagenesis and screening were described previously (Zhang and van Nocker 2002). The *vip3* mutant was backcrossed three times in succession to wild-type plants before phenotypic analysis. Phenotypic analysis of other *vip* mutants was performed with progeny derived from a backcross of M_2 plants to wild type. For genetic complementation analysis, mutants were grown at 18°, under which conditions all mutants were fertile.

Positional cloning of the *VIP3* gene utilized F_2 progeny of a single F_1 individual derived from a cross between *vip3* and introgression line L*er*:FRI^{SF2}:FLC^{SF2}. Bulked-segregant analysis was performed with 24 F_2 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 (http://www.arabidopsis.org/cereon/index.html; courtesy of S. Rounsley) and noted in Figure 3.

Molecular techniques: For use as probes in DNA gel blotting, BAC DNA was purified from 250-ml bacterial cultures using a commercially available kit (QIAGEN, Valencia, CA). For PCR purposes, DNA was prepared from plant tissues using the CTAB-based method described by Lukowitz *et al.* (2000). DNA and RNA gel blotting was performed essentially as previously described (Zhang and van Nocker 2002). For detection of *VIP3* RNA, the probe was a DNA corresponding to the entire *VIP3* coding region, amplified from flower-derived cDNA using primers F27B13.7FBam (5′-AAAGGATCCATGA AACTCGCAGGTCTGAAATCG-3′) and F27B13.7RBam2 (5′-AAAGGATCCGAATTGTTCATGAGTAATCATAGAGC-3′). For detection of *FLC* RNA, the probe was as described by Zhang and van Nocker (2002). For molecular complementation of the *vip3* mutation, an ~6.4-kb *Bam*HI fragment derived from BAC F27B13 was ligated into the *Bam*HI site of vector pPZP:BAR (Zhang and van Nocker 2002) and introduced into wild-type plants through floral dipping. This DNA contained the entire predicted transcriptional units At4g29830 (*VIP3*) and At4g29820, as well as part of transcriptional units At4g29840 and At4g29810. Several independent T1 lines were crossed to the *vip3* mutant, herbicide-resistant progeny from these crosses were allowed to self-pollinate, and the resulting progeny were again subjected to herbicide selection. All *vip3*-like progeny were found to be herbicide sensitive. PCR analysis of several wild-type-like progeny indicated homozygosity for the *vip3* allele and presence of the transgene (data not shown).

For overexpression or antisense expression of *VIP3* in transgenic plants, the *VIP3* coding and 3′ untranslated region was amplified from genomic DNA using primers F27B13.7FBam and F27B13.7RBam (5′-AAAGGATCCAATGCCATCCCTGA CATGGCTTGC-3′). These primers incorporate a *Bam*HI restriction endonuclease site into both termini. The PCR products were ligated into vector pGEM-T (Promega, Madison, WI), the resulting construction was subjected to digestion with *Bam*HI, and the fragment containing the *VIP3* coding and 3′ region was ligated into the *Bam*HI site of vector pPZP:*BAR*:35S (Zhang and van Nocker 2002). Ligation products were obtained that contained the *VIP3* fragment in both forward (sense) and reversed (antisense) orientation. These were introduced into Agrobacterium strain ABI, and the resulting strains were used to infect wild-type plants through floral dipping.

For immunological studies, recombinant, hexahistidine-tagged, full-length VIP3 protein was expressed in *E. coli* and purified using Ni^{2+}-affinity chromatography and a commercially available kit (Novagen; His-Bind) according to the manufacturer's instructions. This purified protein was used to generate anti-VIP3 sera in rabbits. For immunoblotting, plant protein extracts were prepared by grinding tissues under liquid nitrogen, adding the frozen tissue powder to sample buffer containing 4% SDS (Laemmli 1970), incubating at 100° in a boiling water bath, and clarifying by centrifugation for 5 min at 12,000 × *g*. Immunoblotting was done as described by Harlow and Lane (1988), using PVDF membranes (Bio-Rad, Richmond, CA) blocked with Tween-20 in phosphate-buffered saline and alkaline-phosphatase-labeled goat anti-rabbit IgGs (Bio-Rad).

Sequence analysis: WD motifs in the VIP3 protein were identified using the Protein Sequence Analysis server (http://bmerc-www.bu.edu/psa/index.html) at the BioMolecular Engineering Research Center, Boston University. 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).

RESULTS

Identification and genetic analysis of the *VIP3* locus: To identify new floral repressors important for regulation of *FLC*, we extensively mutagenized the late-flowering, vernalization-responsive (winter-annual) genetic background Col:FRI^{SF2} (hereafter referred to as wild-type) using fast neutrons, T-DNA, and EMS and identified individuals that flowered very early independently of a vernalizing cold exposure. Several early flowering individuals were recovered that displayed defects in floral morphology similar to that described for the previously identified *vip4* mutants and that were found to be nonallelic with *vip4* (see below). One of these mutants, designated *vip3*, was selected for further study (Figure 1A). To determine if the defect conferred by the *vip3* mutation could be in a flowering-repressive mechanism involving *FLC*, we evaluated *FLC* expression by RNA gel blotting of seedling RNAs. In contrast to wild-type plants, *FLC* RNA expression was not detectable in the *vip3* mutant, even with phosphorimaging and extended exposures (Figure 1B).

In addition to the defect in flowering time (see below), *vip3* plants exhibited several other defects in growth and development when grown under standard conditions. Specifically, rosette leaves of *vip3* plants were smaller than those of wild-type plants, and overall plant size was reduced (Figure 1A, c). In addition, flowers of *vip3* plants exhibited abnormalities of organs in the outer three whorls (Figure 1A, h). Sepals typically had irregular, translucent margins, and lateral sepals were

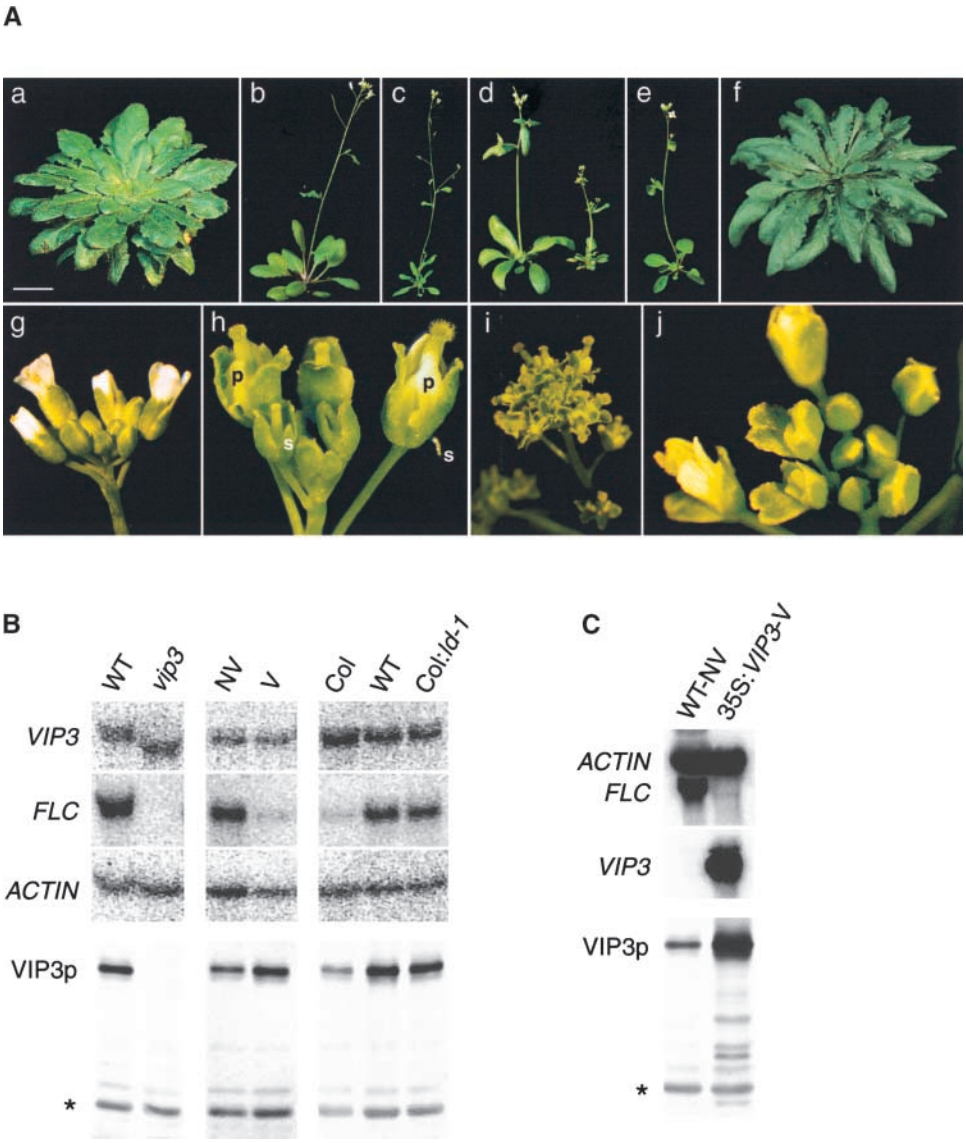

FIGURE 1.—Phenotype of the *vip3* mutant and analysis of *VIP3* expression. (A) Whole-plant and floral phenotypes of wild-type plants, the *vip3* mutant, and *VIP3* transgenic plants. All plants in a–f are shown at the same scale. (a) Wild-type plant grown in the absence of cold. Bar, 25 mm. (b) Wild-type plant grown after a vernalizing cold treatment. (c) *vip3* mutant plant grown in the absence of cold. (d) Ecotype L*er* wild-type (left) and a L*er*::*vip3* introgression line (right). (e) Representative transgenic plant expressing *VIP3* antisense RNA, grown in the absence of cold. (f) Transgenic *vip3* plant containing an introduced copy of the wild-type *VIP3* gene. (g) Inflorescence from a wild-type plant. The more basal flowers were removed. (h) A *vip3* mutant inflorescence with flowers showing reduced or filamentous sepals (s) and petals (p). (i) Inflorescence from the L*er*::*vip3* introgression line. (j) Inflorescence from a representative transgenic plant expressing *VIP3* antisense RNA. (B) Gel-blot analysis of *FLC* and *VIP3* RNA expression and immunoblot analysis of VIP3 protein (VIP3p) abundance in 10-day-old wild-type (WT) and *vip3* mutant seedlings, in 2-week-old nonvernalized (NV) or vernalized (V) wild-type plants, and in 2-week-old Col, WT, and *ld-1* (Col:*ld-1*) mutant plants. (C) Gel-blot analysis of *FLC* and *VIP3* RNA expression and immunoblot analysis of VIP3 protein abundance in nonvernalized wild-type plants (WT-NV) and a representative vernalized 35S:*VIP3* plant. For RNA blots, hybridization of RNAs with an actin probe is shown to indicate relative abundance of mRNA in each lane. For immunoblots, a weakly immunoreactive protein species is indicated (asterisk) to show relative abundance of total protein in each lane.

always reduced in size. Petals were also reduced in size and were often variable in number; whereas wild-type flowers have four petals, *vip3* flowers had up to six. Stamens were typically decreased in number from six to four or five. Organs in the outer three whorls were also often replaced by filamentous structures. The gynoecium was morphologically normal, but slightly reduced in size. Flowers were typically male sterile, and self-pollination was rare. When plants were grown at a lower temperature (18°), these floral defects were attenuated, and plants were typically fertile (data not shown). The *vip3* mutation conferred essentially identical pleiotropy when introgressed into the commonly used L*er* ecotype (Figure 1A, d and i).

Similar to wild-type plants, heterozygous *VIP3/vip3* plants resulting from a backcross between *vip3* and wild-type plants flowered very late under photoperiodically inductive (long-day) conditions in the absence of cold. In addition, none of the phenotypic defects described above for the *vip3* mutant were apparent in *VIP3/vip3* plants (data not shown). These observations indicate that the *vip3* mutation is effectively recessive. In the progeny of a *VIP3/vip3* plant, mutant individuals were found with a frequency expected for Mendelian segregation of a single recessive locus (data not shown). In addition, analysis of the progeny of reciprocal crosses between a wild-type plant and a *VIP3/vip3* plant indicated that the *vip3* mutant allele was transmitted through both male and female gametes with a frequency similar to that of the wild-type allele (data not shown).

Flowering response of the *vip3* mutant: To better define the position of *VIP3* with respect to flowering pathways involving *FLC*, we evaluated the effects of photoperiod, extended cold, and light quality on the flow-

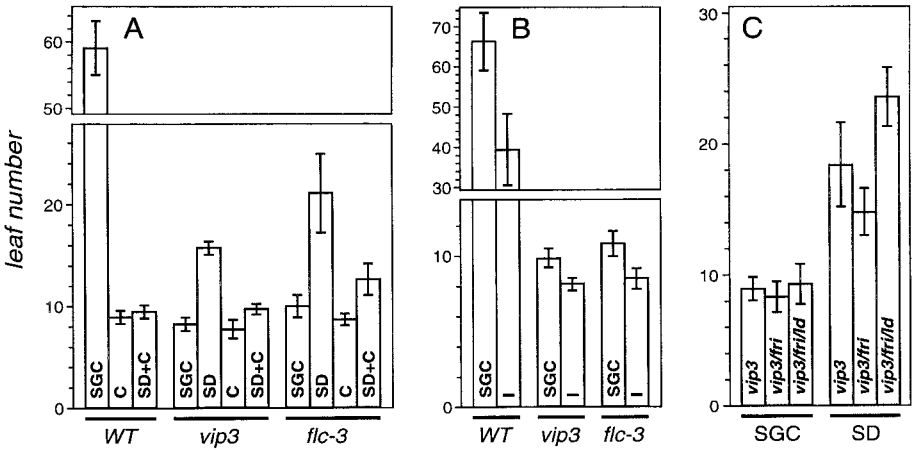

FIGURE 2.—Flowering time of genotypes used in this study as affected by photoperiod and cold treatment (A), light quality (B), or photoperiod (C). Flowering time was evaluated under standard growth conditions (SGC; long-day photoperiods, without cold treatment and with fluorescent lighting) or SGC modified as follows: SD, short-day photoperiods; C, 70-day cold treatment; and I, incandescent lighting. At least 10 plants of each genotype were evaluated in each condition. Error bars indicate the standard deviation. Individual panels reflect the results of independent experiments. Flowering time was measured as the total number of leaves (vegetative nodes) produced on the primary stem. Scale varies along the *y*-axis. Wild-type plants grown in short-day photoperiods without cold did not flower during the course of this experiment.

ering response of the *vip3* mutant relative to that of wild-type plants and plants carrying a null mutation in *FLC* (Figure 2, A and B). When grown in the absence of cold, wild-type plants produced ~67 leaves under long-day conditions but did not flower under short-day (noninductive) conditions, even after producing >100 rosette leaves. We found that when given an extended (70 days) cold treatment, the photoperiodic response of wild-type plants was effectively eliminated, and plants flowered after producing ~10 or 11 leaves irrespective of photoperiod (Figure 2A). This is consistent with current models of flowering where vernalization, acting through suppression of *FLC*, can bypass the lack of floral promotion from inactivity of the photoperiodic pathway (SIMPSON and DEAN 2002). Both the *flc* null mutant and the *vip3* mutant exhibited strong photoperiodic responses when grown in the absence of cold and still showed slight photoperiodic responses even after being given a 70-day cold treatment (Figure 2A).

When grown under photoperiodically inductive conditions, the vernalization response of winter-annual types of Arabidopsis appears saturated after ~40 days of cold; *i.e.*, longer cold periods have no further flowering-promotive effects (LEE and AMASINO 1995). This probably reflects the ability of the photoperiodic pathway to partially bypass repression of flowering by *FLC*, because much longer (*e.g.*, ~70 days) cold periods are necessary to saturate the vernalization response when flowering is evaluated in noninductive photoperiods (LEE and AMASINO 1995). Under short-day conditions, we found that *vip3* plants grown in the absence of cold flowered appreciably later (17.8 ± 0.9 leaves) than wild-type plants given a 70-day cold treatment (10.7 ± 0.9 leaves; Figure 2). A 70-day cold treatment reduced flowering time of vip3 plants to that of vernalized wild-type plants (Figure 2). The observation that cold effectively promotes flowering in *vip3* plants suggests that, if *vip3* is a null mutation, vernalization could promote flowering at least partially independently of *VIP3*.

Wild-type plants flowered much earlier (44.7 ± 10.2 leaves) when grown under far-red-enriched light supplied by incandescent bulbs than when grown under the fluorescent lights used in our standard growth conditions (75.2 ± 8.2 leaves; Figure 2B), and this is consistent with previous observations that far-red-enriched light promotes flowering in genotypes that strongly express *FLC* (BAGNALL 1993; LEE and AMASINO 1995). We found that *flc* mutant plants flowered earlier when grown under incandescent lighting (9.6 ± 0.9 leaves) relative to fluorescent lighting (12.2 ± 1.1 leaves; Figure 2B), suggesting that promotion of flowering associated with light quality is mediated at least partly independently of *FLC*. The *vip3* mutant plants also flowered earlier under these conditions (9.2 ± 0.6 leaves) than under standard growth conditions (11.1 ± 0.8 leaves), and the net reduction in flowering time was similar to that observed for the *flc* mutant. This observation suggests that mechanisms that promote flowering in response to light quality are intact in the *vip3* mutant.

Under all conditions evaluated, *vip3* mutant plants flowered earlier than *flc* mutants (Figure 2, A and B). This was most apparent when plants were grown in short-day photoperiods, irrespective of cold. This observation indicates that *VIP3* has an additional flowering-repressive role that is mediated outside of its positive regulation of *FLC*.

Interactions with *FRI* and *LD*: One of several potential positions of the *VIP3* gene within the regulatory hierarchy of flowering is as a negative regulator of the activity of the autonomous pathway, a function that has been proposed for *FRI* (above). If this were the case, then loss of *VIP3* function would not be expected to suppress the late flowering associated with loss of autonomous pathway activity. To test this, we evaluated the epistatic interactions between *VIP3* and the autonomous pathway gene *LD*. We introduced the *vip3* mutation into the Col::*ld-1* genetic background, which carries strong loss-of-function alleles in both *FRI* and *LD*. Col::*ld-1* plants

behave as winter annuals, because loss of *LD* activity leads to derepression of *FLC*; these plants were otherwise phenotypically indistinguishable from wild-type plants (data not shown). We found that *vip3/fri/ld* triple-mutant plants were phenotypically similar to *vip3* plants, exhibiting aberrant floral morphology and reduced plant size (data not shown). Under long-day conditions, there was no apparent difference in flowering time between *vip3* plants and the *vip3/fri/ld* triple mutant (Figure 2C). Under short-day conditions, however, the *vip3/fri/ld* triple mutant flowered notably later (26.7 ± 2.9 leaves) than *vip3* plants (20.8 ± 3.8 leaves; Figure 2C). That the *vip3* phenotype was predominantly epistatic to the late-flowering *ld* phenotype indicates that *VIP3* is unlikely to function as an upstream regulator of the autonomous pathway and that it has a function that is distinct from that of *FRI*. The observation that this epistasis was incomplete also suggests that *VIP3* could function in a pathway that is distinct from the autonomous pathway mechanism involving *LD*. A caveat to this analysis is that the incomplete epistasis observed could potentially result from weak function of *VIP3*, if the *vip3* mutation were not null.

To determine if *FRI* has any flowering-repressive effect in a *vip3* genetic background, we evaluated the effect of the strong loss-of-function fri^{Col} allele on flowering time of *vip3* plants. When grown under long-day photoperiods, there was no significant difference in flowering time between *vip3* single mutants and *vip3/fri* double mutants (Figure 2C). Under short-day photoperiods, *vip3/fri* double mutants flowered marginally earlier (16.7 ± 2.2 leaves) than *vip3* single mutants (20.8 ± 3.8 leaves; Figure 2C). Thus, with respect to flowering time, the effect of the *vip3* mutation was strongly epistatic to the effect of *FRI*. Mutants lacking both *VIP3* and *FRI* were otherwise phenotypically similar to *vip3* single mutants, exhibiting aberrant floral morphology and reduced plant size (data not shown).

Positional cloning of the *VIP3* gene: Through genetic mapping, we localized the *vip3* mutation to an ~2.4-Mb region of the lower arm of chromosome IV, represented by three overlapping BAC clones (Figure 3A). Subsequently, we analyzed genomic DNA from *vip3* and wild-type plants by gel blotting using these three BACs as probes. This approach resulted in the indication of an insertion within the predicted coding region of a transcriptional unit designated At4g29830 by the Arabidopsis Genome Initiative (AGI; Figure 3A). Further analysis using inverse PCR and sequencing indicated that the insertion was associated with the translocation of up to ~320 kb, a possibly contiguous sequence from a proximal region of chromosome IV (data not shown). We found that, in the *vip3* mutant, RNAs hybridizing with an At4g29830 probe accumulated to detectable levels, but were shorter than RNAs seen in wild-type plants, suggesting that the insertion in the *vip3* mutant resulted in a truncation of the At4g29830 gene (Figure 1B). DNA and RNA gel-blot analyses of the adjacent genes, At4g29820 and At4g29840, in the *vip3* mutant revealed that both genes were intact and expressed to levels similar to those seen in wild-type plants (data not shown). Using immunoblotting and antisera generated against recombinant At4g29830 protein, we observed a highly reactive protein species in wild-type plant extract approximating the predicted size of the At4g29830 protein (Figure 1B). This protein was not detectable in equivalent extracts from *vip3* plants (Figure 1B). Also, there were no immunoreactive protein species of larger or smaller molecular mass unique to *vip3* extracts (Figure 1B and data not shown), suggesting that any aberrant protein produced from the At4g29830 gene in *vip3* plants is unstable. A query of Arabidopsis expressed sequence tag databases resulted in the identification of six independent cDNAs corresponding to the At4g29830 gene. These cDNAs collectively defined a transcribed region and intron/exon structure that is consistent with that predicted by the AGI and with the size of At4g29830 RNAs as determined by gel blotting (Figure 3A). RNA gel-blot analysis and immunoblot analysis indicated that At4g29830 RNA and protein are expressed throughout the plant (data not shown).

To determine if disruption of At4g29830 was the lesion causing the *vip3* phenotype, we performed molecular complementation in transgenic plants using an ~6.4-kb DNA containing the entire At4g29830 transcriptional unit. Because the *vip3* mutant was predominately male sterile when grown under standard conditions and was therefore incompatible with the standard floral-dip method of plant transformation (Clough and Bent 1998), we first introduced the transgene into wild-type plants and then introduced the transgene into the *vip3* mutant through crossing. Several independent lines were generated that were homozygous for the translocation mutation and contained at least one copy of the *VIP3* transgene. These plants were phenotypically indistinguishable from wild-type plants, flowering extremely late in the absence of cold and producing morphologically normal flowers (Figure 1A, f and data not shown). On the basis of these data and the observation that antisense expression of At4g29830 resulted in a phenocopy of the *vip* phenotype (below), we concluded that At4g29830 is the *VIP3* gene.

On the basis of annotation provided by the AGI, *VIP3* encodes a 321-amino-acid protein that is composed almost entirely of seven repeats of a motif designated the Trp-Asp (WD) motif (also known as the WD-40 repeat; Neer *et al.* 1994; Smith *et al.* 1999; Figure 3B). No closely related genes exist in the Arabidopsis genome, and the predicted VIP3 protein does not show extensive sequence homology with any protein cataloged in current protein databases.

***VIP3* expression:** Genetic epistasis analysis (above) indicated that *VIP3* could function downstream from the strong *FLC* regulators *FRI* and *LD*. To determine if

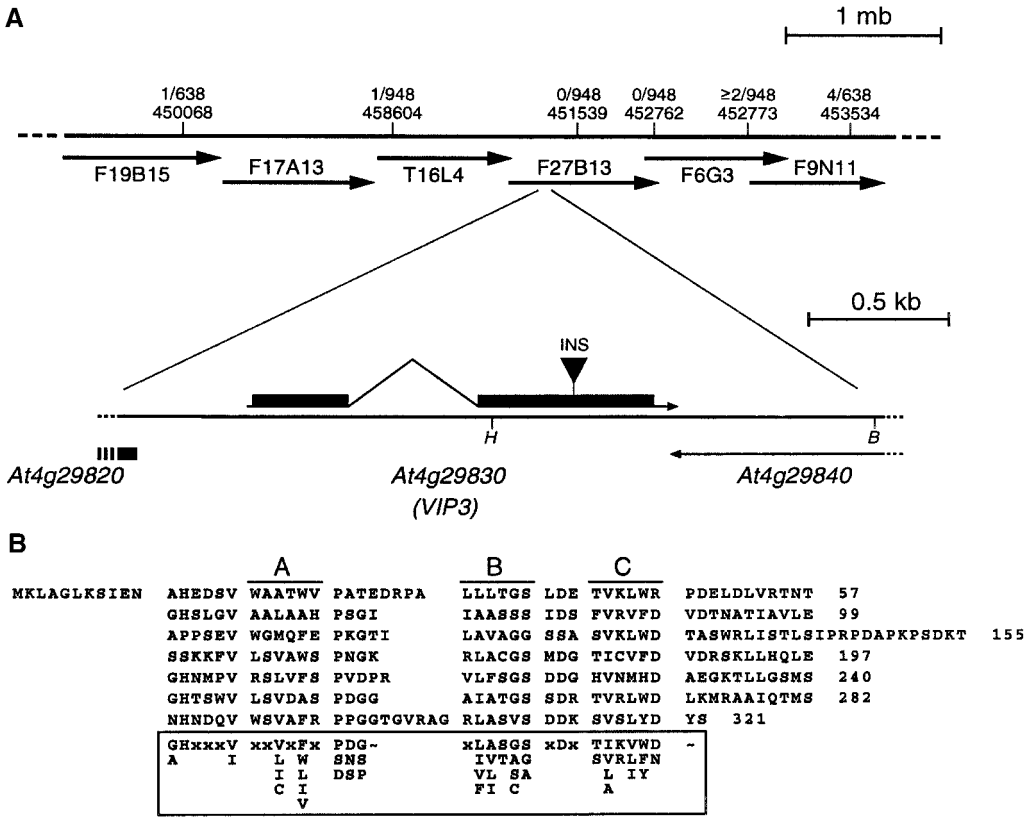

FIGURE 3.—(A) Region of chromosome IV encompassing BAC F27B13 containing the *VIP3* (At4g29830) gene. Molecular markers used in mapping are shown (top), with genetic distance (recombinations / chromosomes analyzed) between the marker and *vip3* mutation identified. The position and orientation of BAC clones is indicated. An enlargement of the region containing the *VIP3* (At4g29830) gene is shown (bottom). Lines with arrows indicate the orientation and extent of RNA transcripts, as determined from analysis of cDNAs present in current databases. No cDNAs were identified for At4g29820. Open reading frames as predicted by the AGI are shown as solid rectangles. The position of the insertion in the *vip3* mutant is shown (INS). Restriction sites that were used in DNA gel-blot analysis to delineate the region containing the mutation are shown (*H*, *Hin*dIII; *B*, *Bam*HI). (B) Amino acid sequence of the VIP3 protein with WD repeats aligned. The consensus sequence that defines the alignment of the repeats is enclosed in a box. This consensus sequence includes those residues that most frequently occur at a specific position [SMITH *et al.* (1999), modified as described by T. F. Smith (http://bmerc-www.bu.edu/wdrepeat/)]. The letter x signifies that any amino acid can be found at the position. The symbol ~ signifies that additional amino acids are typically present at the position. The position of three antiparallel β-strands, here labeled A, B, and C, is based on the structure determined for the Gβ protein. A fourth β-strand found in WD motifs, strand D, is not strongly conserved at the amino acid sequence level and is not indicated.

the promotive activity of *FRI* on *FLC* expression might be mediated through activation of *FLC* by *VIP3*, we compared *VIP3* RNA and protein abundance in wild-type plants with that in the Col ecotype (lacking strong *FRI* activity). Likewise, to determine if *VIP3* might mediate the derepression of the *FLC* gene due to loss of *LD* activity, we evaluated *VIP3* RNA and protein levels in the *ld-1* mutant. Although loss of *FRI* or *LD* activity resulted in obvious differences in *FLC* RNA expression, no effect on *VIP3* expression was apparent (Figure 1B). To determine if the repressive effect of cold on *FLC* expression might be mediated through loss of *VIP3* activity, we evaluated *VIP3* RNA and protein levels in vernalized and nonvernalized wild-type plants. In both situations, *VIP3* was expressed to similar levels (Figure 1B). These findings suggest that modulation of *VIP3* RNA or protein levels is unlikely to be involved in the regulation of *FLC* by *FRI*, the autonomous pathway, or cold.

Constitutive and antisense expression of *VIP3* in transgenic plants: To study the potential effects of manipulated expression of *VIP3* on growth and development, we engineered transgenic plants in which the wild-type genomic copy of *VIP3* was expressed in either sense or antisense orientation, under control of the constitutive CaMV 35S promoter. For both the sense (35S:*VIP3*) and the antisense (*VIP3*-AS) strategies, at least 150 transgenic plants were recovered. For the *VIP3*-AS strategy, self-pollinated offspring from infiltrated plants (designated T1 plants) were grown without a vernalizing cold treatment. Approximately one-half of *VIP3*-AS plants surviving selection flowered very early, with as few as 5 rosette leaves (Figure 1A, e). In contrast, nonvernalized wild-type plants grown under similar conditions produced at least 60 rosette leaves without flowering (Figure 1A, a). In addition, the typical early flowering *VIP3*-AS plants were smaller than wild-type plants and produced morphologically abnormal flowers similar to those seen on *vip3* plants (Figure 1A, e and j). For the 35S:*VIP3* strategy, a population of T1 plants was grown without a vernalizing cold treatment. Similar to nonvernalized wild-type plants, the great majority of these plants flowered extremely late or did not flower during the course of this experiment. Analysis of *VIP3* RNA and protein levels in leaf tissues of eight of these late-

flowering plants indicated that all expressed the 35S:*VIP3* transgene to high levels relative to wild-type nontransgenic plants (data not shown). The few very early flowering plants observed in this population possibly resulted from transgene-associated suppression of the endogenous *VIP3* gene, as VIP3 protein was not detectable in leaf tissues of these plants (data not shown).

To determine if constitutive expression of *VIP3* could overcome the repressive effect of cold on *FLC* expression, we analyzed another population of *35S:VIP3* T1 plants grown after being subjected to a vernalizing cold treatment. In this population of ~250 individuals, all plants flowered very early, and there was no large variation in flowering time among the plants (data not shown). *VIP3* was expressed to high levels in several of these plants, as determined by RNA gel-blot and immunoblot analyses of leaf tissues (Figure 1C). Even in these *VIP3*-expressing plants, *FLC* expression was not detectable (Figure 1C). These findings indicate that *VIP3* is probably insufficient to activate *FLC* in vernalized plants.

***VIP3* is a member of a class of functionally related genes in Arabidopsis:** The phenotype of the *vip3* mutant was similar to that of plants with mutations in the previously identified flowering-time gene *VIP4* (Zhang and van Nocker 2002). For example, *vip4* mutants do not express detectable levels of *FLC*, flower earlier than an *FLC* null mutant, and exhibit defects in floral morphology in whorls 1–3 (Zhang and van Nocker 2002). The observation that several *vip3/vip4*-like mutations identified through our genetic screens were nonallelic to either *VIP3* or *VIP4* (see below) indicated that a class of gene with similar roles in flowering timing and floral development could exist in Arabidopsis. To attempt to define the extent of this potential gene class, we mapped all of the seven *vip3/4*-like mutations recovered in our screens to a limited region of the genome, and those plants harboring mutations on the same chromosome were subjected to genetic complementation analysis (Tables 1 and 2). In addition to *VIP3* and *VIP4*, five new loci were identified, which we tentatively designated *VIP1*, *VIP2*, *VIP5*, *VIP6*, and *VIP7* (Tables 1 and 2; Figure 4). All of the mutants exhibited early flowering, reduced plant size, and floral defects in whorls 1–3 similar to those described for *vip3* above. In all cases these phenotypes cosegregated with early flowering in the small mapping populations and with a frequency expected for Mendelian segregation of a single, recessive locus (Figure 4 and data not shown). When evaluated under short-day photoperiods in the absence of cold, most of these mutants flowered as early as, or earlier than, an *flc* null mutant (Figure 4E). The exception was *vip2*, originating in an EMS-mutagenized population, which flowered slightly later (Figure 4E). Analysis of *FLC* RNA levels in the mutants revealed that in all cases except for *vip2*, this early flowering was associated with loss of detectable *FLC* expression (Figure 4, B and C and data not shown). For the *vip2* mutant, *FLC* expression was detectable using phosphorimaging and extended exposures (data not shown). None of the mutations mapped to previously described flowering-time genes, with the exception of *VIP2* and *VIP5*, which are located within a broad region described for *EARLY FLOWERING IN SHORT DAYS* (*EFS*; Soppe *et al.* 1999).

TABLE 1

Approximate map positions of *VIP* loci

Locus	No. alleles	Chromosome	Marker	Recombination
VIP1	1	I	nga280	0/100
VIP2	1	I	nga111	11/118
VIP3	1	IV	(Cloned)[a]	(Cloned)[a]
VIP4	3	V	(Cloned)[b]	(Cloned)[b]
VIP5	1	I	nga111	14/104
VIP6	3	II	ciw3	12/164
VIP7	1	V	ciw10	10/136

The molecular markers used and recombination between the mutation and the marker (recombinations/chromosomes analyzed) are indicated.

[a] This report.

[b] Zhang and van Nocker (2002).

To help clarify the potential relationships among these genes, we analyzed *VIP3* and *VIP4* mRNA levels and VIP3 protein abundance in all of the *vip* mutant backgrounds. In the *vip1*, *vip2*, *vip5*, *vip6-3*, and *vip7* backgrounds, *VIP3* and *VIP4* RNA levels and VIP3 protein abundance were similar to that seen in wild-type plants (Figure 4, B–D and data not shown). In addition, *VIP3* RNA and protein was expressed to apparent wild-type levels in plants carrying the strong *vip4-3* mutation, and *VIP4* RNA was expressed to apparent wild-type levels in the *vip3* mutant (Figure 4, C and D). These findings suggest that, if these genes function in a pathway involving *VIP3* or *VIP4*, they probably do not act as direct regulators of *VIP3* or *VIP4*.

DISCUSSION

Current models of flowering propose that *FLC* is regulated through several mechanisms, including the autonomous pathway, *FRI*, and vernalization (Simpson and Dean 2002). In addition, both genetic and molecular studies indicate that *FLC* is weakly regulated by genes operating outside of these pathways, as a result of poorly defined "feedback" mechanisms (Koornneef *et al.* 1998a; Rouse *et al.* 2002). *VIP3* encodes a strong activator of *FLC*, as *FLC* RNA expression appears to be abolished in the *vip3* mutant. Importantly, *FLC* expression is apparently ubiquitous in wild-type plants and does not appear to be developmentally regulated (Sheldon *et al.* 1999), indicating that *VIP3* acts in a more direct manner. *VIP3* is also expressed throughout the plant, similar to *FLC* (Sheldon *et al.* 1999), and this is consistent with a role for *VIP3* as a direct activator of *FLC*.

As an activator of *FLC*, *VIP3* functions in a manner distinctly different from *FRI*, which has been proposed

TABLE 2

Complementation testing of *vernalization independence* mutants

	Male									
Female	FN124	EMSH1	X2	FS97	FN212	D2	U1	T48	FS7	FN128
FN124 (*vip1*)		+				+				
EMSH1 (*vip2*)	+					+				
X2 (vip4-1)				−	−					+
FS97 (*vip4-2*)			−		−					+
FN212 (*vip4-3*)			−	−						+
D2 (*vip5*)	+	+								
U1 (vip6-1)								−	−	
T48 (vip6-2)							−		−	
FS7 (vip6-3)							−	−		
FN128 (*vip7*)			+	+	+					

All mutant strains were homozygous. Symbols indicate wild-type (+) or mutant (−) phenotype of F_1 progeny from the crosses indicated. In all cases where crosses produced mutant F_1 progeny, only mutant individuals were observed in the corresponding F_2 progeny. Selection for crosses to be analyzed was on the basis of map position determined for the respective mutations (see text).

to limit that activity of the autonomous pathway (Zhang and van Nocker 2002). This was evident by the epistasis analysis employing a strong mutation in the autonomous pathway gene *LD*. The derepression of *FLC* and late flowering conferred by the *ld* mutation is effectively epistatic to loss of *FLC* activation and early flowering conferred by the strong loss-of-function fri^{Col} allele. This effect was largely suppressed when the *vip3* mutation was introduced into the ld/fri^{Col} background. This indicates that *VIP3* probably does not act to limit the activity of *LD*. However, this epistasis was incomplete, and the small inhibitory effect caused by loss of *LD* function in a *vip3* background could suggest that *vip3* functions at least partly independently of *LD*. In addition, we found that an active *FRI* allele marginally delays flowering in a *vip3* background, suggesting that *VIP3* might have *FRI*-independent functions. These conclusions are dependent on the *vip3* mutation creating a total loss of function of the *VIP3* gene. Although full-length *VIP3* RNA or protein was not detectable in the *vip3* mutant in our experiments, it is possible that *VIP3* is still expressed at a very low level.

The lack of effect of disruption of *FRI* or *LD* on *VIP3* RNA or protein expression suggests that modulation of *VIP3* expression is unlikely to be involved in the regulation of *FLC* by *FRI* or *LD*. Likewise, because *VIP3* RNA and protein are expressed to similar levels in vernalized and nonvernalized plants, *VIP3* is unlikely to be a direct regulator of the vernalization response. Possibly, regulation of *VIP3* by these factors is carried out through modification of protein activity or within a small spatial domain. However, the simple observation that the developmental pleiotropy conferred by the *vip3* mutation is not apparent in a *fri* null mutant, where the autonomous pathway is actively suppressing *FLC*, or in vernalized plants, also suggests that *VIP3* retains activity under these circumstances. Thus, our data are most consistent with *VIP3* acting outside of mechanisms involving *FRI* or *LD*.

A mechanism of *FLC* regulation in which *VIP3* participates could be a major target of the vernalization pathway. This is suggested by the observation that both *vip3* and vernalization affect flowering predominately through *FLC*, but also through *FLC*-independent mechanisms. Our findings that a long cold treatment slightly accelerates flowering of the *vip3* mutant and that *vip3* plants flower slightly later than vernalized wild-type plants could indicate that vernalization is mediated at least partly outside of *VIP3* activity. However, a slight vernalization response could be mediated by weak *VIP3* activity in *vip3* plants. These possibilities can be resolved only through the identification and analysis of an unambiguous *vip3* null mutation.

VIP3 encodes a protein containing WD motifs. The WD motif is found in a large variety of proteins that do not share any obvious function (Neer *et al.* 1994). The crystal structure of the well-known WD-repeat protein Gβ shows that each of the seven WD motif units takes the form of four antiparallel β-strands, with the seven repeated WD motifs forming a symmetrical structure termed a β-propeller (Smith *et al.* 1999). A distinctive feature of the VIP3 protein is a 13-residue extension within the region predicted for strand D of repeat IV. The analogous region of Gβ takes the form of a loop comprising the exterior surface of the propeller structure, and additional amino acids at this position may comprise an independently folded domain that would protrude from the structure. VIP3 appears to lack extensive amino- or carboxyl-terminal domains outside of the potential β-propeller, suggesting that it could act exclusively in the context of a molecular scaffold.

We formerly identified the Arabidopsis *VIP4* gene, an *FLC* activator that encodes a highly hydrophilic protein with similarity to the Leo1 protein from *Saccharomyces*

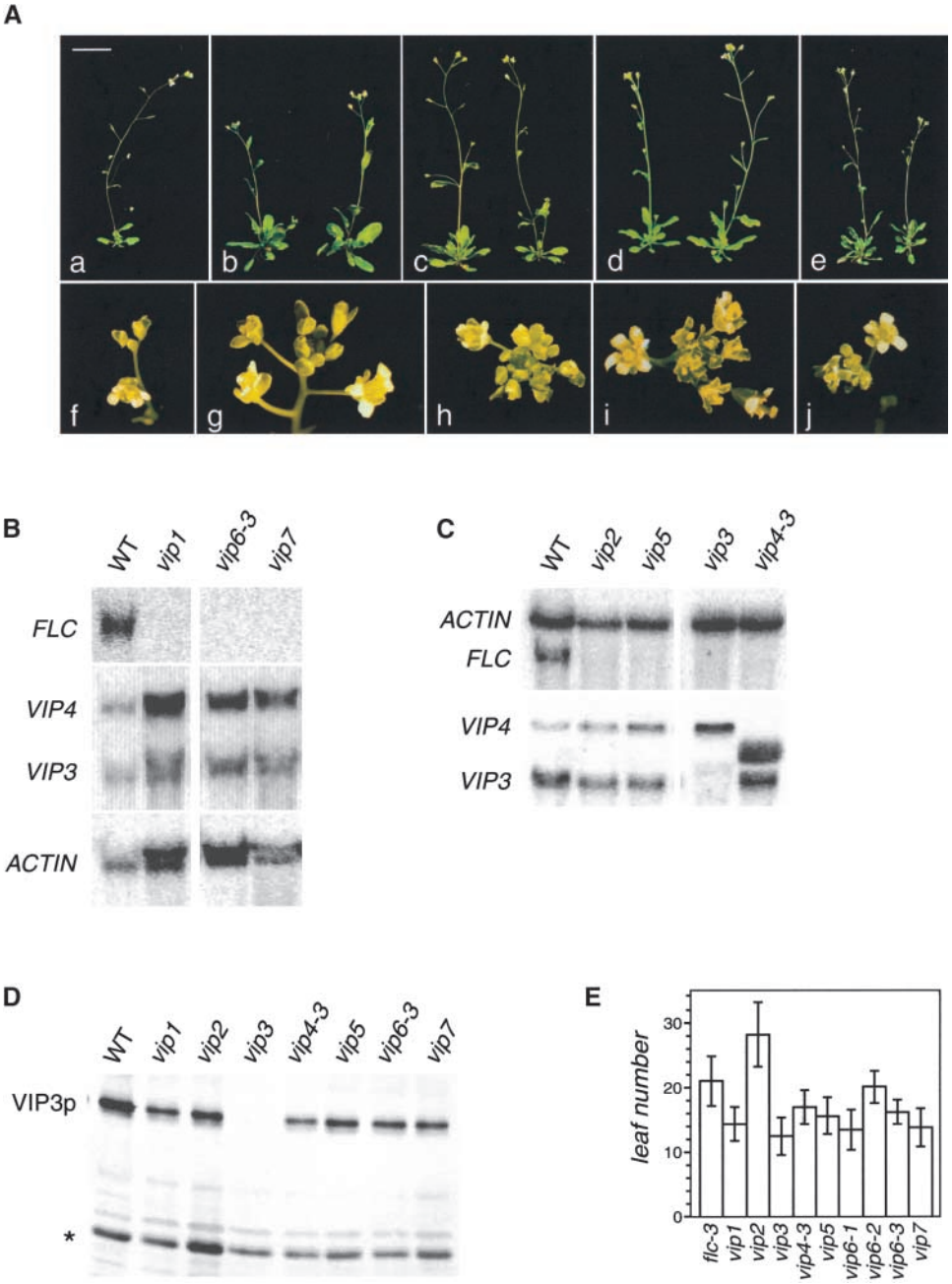

FIGURE 4.—Characterization of Arabidopsis *vip* mutants. (A) Nonvernalized *vip* mutant plants showing reduced size and early flowering (a–e) and floral defects (f–j). Shown are *vip1* (a and f), *vip2* (b and g), *vip5* (c and h), *vip6-3* (d and i), and *vip7* (e and j). All plants in a–e are shown at the same scale. Bar in a, 25 mm. (B and C) RNA gel blots showing *FLC*, *VIP3*, and *VIP4* expression in wild-type (WT) plants and in other *vip* mutants. The left and right panels of B and C were assembled from the same images. (D) Immunoblot analysis of VIP3 protein expression in WT plants and in *vip* mutants. A weakly immunoreactive protein species is indicated (asterisk) to show relative abundance of total protein in each lane. (E) Flowering time (total leaf number) of the null *flc-3* mutant and *vip* mutants grown in short-day photoperiods without cold. At least 15 plants of each genotype were evaluated. Error bars indicate the standard deviation.

cerevisiae and similar proteins from Drosophila and *Caenorhabditis elegans* (ZHANG and VAN NOCKER 2002). Leo1 is involved in the expression of a small subset of yeast genes, as a component of the Paf1 transcriptional regulator, which may represent a transcriptional endpoint of protein kinase C-mitogen-activated protein kinase signaling (MUELLER and JAEHNING 2002). On the basis of the phenotypic similarity between the *vip3* and *vip4* mutants, the observation that the *VIP4* gene exhibits epistatic relationships with *FRI* and *LD* that are similar to that seen for *VIP3*, and our observations that the *vip4-1* mutation does not obviously enhance the phenotype of *vip3* plants (our unpublished results), it is likely that the *VIP3* and *VIP4* genes act in close functional proximity. The relationship between *VIP3* and *VIP4* at the molecular level is not known, but our results suggest that it does not involve modulation of RNA expression of either gene or modulation of VIP3 protein abundance.

In addition to *VIP4*, mutations at five other loci create phenotypes that are superficially indistinguishable from that of *vip3*. Although two of the *VIP* loci, *VIP2* and *VIP5*, map roughly to the previously identified flowering-time gene *EFS*, the *vip2* and *vip5* mutants do not exhibit specific pleiotropic phenotypes described for *efs* mutants. For example, *efs* mutants show increased seed dormancy, decreased apical dominance, and normal development of more apical flowers (SOPPE *et al.* 1999), and these phenotypes were not observed in the *vip* mu-

tants. In addition, the specific floral defects seen in the *vip* mutants were not reported in *efs* mutants (Soppe *et al.* 1999). Thus the *VIP* loci probably define a previously unreported group of flowering repressors. In spite of the large numbers of mutagenized plants screened in this study, the screen does not appear to approach saturation, as five of the seven *VIP* loci are defined by only a single allele. Therefore this group could be extensive. Although the relationships among these genes remain largely undefined, evidence presented here indicates that they are unlikely to act to modify *VIP4* RNA, *VIP3* RNA, or protein levels. One possibility is that these genes define components of a protein complex, potentially analogous to the yeast Paf1 transcriptional complex. However, at least VIP3 does not exhibit strong homology with known Paf1 components or with any other yeast protein.

In addition to its early flowering phenotype, *vip3*, *vip4*, and the other *vip* mutants described here display similar defects in floral development. Because plants lacking *FLC* do not display floral defects, the role of these genes in floral development is mediated outside of their regulation of *FLC*. We formerly proposed (Zhang and van Nocker 2002) that *VIP4* may have a floral function similar to that of a class of gene involved in repressing the expression of *AGAMOUS* (*AG*) and/or other floral homeotic genes outside of their typical spatial or temporal domains. We analyzed *AG* RNA abundance in *vip3* fully developed flowers and found that it was elevated substantially (~50%) over wild-type flowers (data not shown) but it remains unclear if this resulted from a direct role in *AG* expression or was merely an indirect effect of altered morphology of *vip3* flowers.

We propose that the *VIP* gene class defines a mechanism involved in multiple developmental processes, including flowering (through activation of *FLC*) and floral development (through interaction with yet-undefined factors). The activity of such a mechanism in specific contexts could be directed by spatial or temporal cues provided by specific auxiliary factors. The functions in plant development that we propose for *VIP3* are similar, but opposite, to those described for the *VRN1* gene (Simpson and Dean 2002). For example, gain-of-function studies suggest that, like *VIP3*, *VRN1* is involved in flowering through both *FLC*-dependent and *FLC*-independent mechanisms and that *VRN1* is also involved in developmental processes apparently unrelated to timing of flowering, including floral development (Levy *et al.* 2002). Thus, these two genes could act in an antagonistic manner. Because the silencing of *FLC* associated with vernalization might involve changes in chromatin environment, one possibility is that a *VIP3* mechanism could maintain *FLC* chromatin in a configuration that is relatively accessible to transcription. In additional developmental contexts, this mechanism may act on other genes subject to chromatin-associated silencing. Further characterization of the *VIP* genes will require the identification of additional regulatory targets and the definition of elements that specify these as targets.

We thank Rick Amasino and Scott Michaels for contributing several mutants (*vip1*, *vip6-3*, and *vip7*) for analysis and Steve Rounsley (Cereon Corporation) for making the Cereon Arabidopsis Polymorphism Collection available. We are indebted to Reginald Ronnigen and the MSU Cyclotron Laboratory for assistance with fast-neutron mutagenesis. This work was supported by the Michigan Agricultural Experiment Station and a U.S. Department of Agriculture National Research Initiative Competitive grant (79-35304-5108) to S.v.N.

LITERATURE CITED

Aukerman, M. J., and R. M. Amasino, 1996 Molecular genetic analysis of flowering time in *Arabidopsis*, pp. 427–434 in *Seminars in Developmental Biology*, Vol. 7, edited by R. M. Amasino. Academic Press, Cambridge, UK.

Bagnall, D. J., 1993 Light quality and vernalization interact in controlling late flowering in *Arabidopsis thaliana* ecotypes and mutants. Ann. Bot. **71:** 75–83.

Chandler, J., A. Wilson and C. Dean, 1996 Arabidopsis mutants showing an altered response to vernalization. Plant J. **10:** 637–644.

Clough, S. J., and A. F. Bent, 1998 Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Gendall, A. R., Y. Y. Levy, A. Wilson and C. Dean, 2001 The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell **107:** 525–535.

Harlow, E., and D. Lane, 1988 *Antibodies—A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hepworth, S. R., F. Valverde, D. Ravenscroft, A. Mouradov and G. Coupland, 2002 Antagonistic regulation of flowering-time gene *SOC1* by *CONSTANS* and *FLC* via separate promoter motifs. EMBO J. **21:** 4327–4337.

Johanson, U., J. West, C. Lister, S. Michaels, R. Amasino *et al.*, 2000 Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. Science **290:** 344–347.

Koornneef, M., C. Alonso-Blanco, H. Blankestijn-de Vries, C. J. Hanhart and A. J. M. Peeters, 1998a Genetic interactions among late-flowering mutants of Arabidopsis. Genetics **148:** 885–892.

Koornneef, M., C. Alonso-Blanco, A. J. M. Peeters and W. Soppe, 1998b Genetic control of flowering time in Arabidopsis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49:** 345–370.

Laemmli, U. K., 1970 Cleavage of structural proteins during the assembly of the head of bacteriaphage T4. Nature **227:** 680–685.

Lee, H., S.-S. Suh, E. Park, E. Cho, J. H. Ahn *et al.*, 2000 The *AGAMOUS-LIKE 20* MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev. **14:** 2366–2376.

Lee, I., and R. M. Amasino, 1995 Effect of vernalization, photoperiod, and light quality on the flowering phenotype of Arabidopsis plants containing the *FRIGIDA* gene. Plant Physiol. **108:** 157–162.

Lee, I., S. D. Michaels, A. S. Masshardt and R. M. Amasino, 1994 The late-flowering phenotype of *FRIGIDA* and mutations in *LUMINIDEPENDENS* is suppressed in the Landsberg *erecta* strain of Arabidopsis. Plant J. **6:** 903–909.

Levy, Y. Y., S. Mesnage, J. S. Mylne, A. R. Gendall and C. Dean, 2002 Multiple roles of Arabidopsis *VRN1* in vernalization and flowering time control. Science **297:** 243–246.

Lukowitz, W., C. S. Gillmor and W.-R. Scheible, 2000 Positional cloning in Arabidopsis. Plant Physiol. **123:** 795–805.

Macknight, R., I. Bancroft, T. Page, C. Lister, R. Schmidt *et al.*, 1997 *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding motifs. Cell **89:** 737–745.

Michaels, S. D., and R. M. Amasino, 1999 *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11:** 949–956.

Michaels, S. D., and R. M. Amasino, 2001 Loss of *FLOWERING*

LOCUS C activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. Plant Cell **13:** 935–941.

Mueller, C. L., and J. A. Jaehning, 2002 Ctr9, Rtf1, and Leo1 are components of the Paf1/RNA polymerase II complex. Mol. Cell. Biol. **22:** 1971–1980.

Neer, E. J., C. J. Schmidt, R. Nambudripad and T. F. Smith, 1994 The ancient regulatory-protein family of WD-repeat proteins. Nature **371:** 297–300.

Rouse, D. T., C. C. Sheldon, D. J. Bagnall, W. J. Peacock and E. S. Dennis, 2002 *FLC*, a repressor of flowering, is regulated by genes in different inductive pathways. Plant J. **29:** 183–191.

Samach, A., H. Onouchi, S. E. Gold, G. S. Ditta, Z. Schwarz-Sommer *et al.*, 2000 Distinct roles of *CONSTANS* target genes in reproductive development of *Arabidopsis*. Science **288:** 1613–1616.

Schomburg, F. M., D. A. Patton, D. W. Meinke and R. M. Amasino, 2001 *FPA*, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. Plant Cell **13:** 1427–1436.

Sheldon, C. C., J. E. Burn, P. P. Perez, J. Metzger, J. A. Edwards *et al.*, 1999 The *FLF* MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11:** 445–458.

Simpson, G. G., and C. Dean, 2002 Arabidopsis, the Rosetta Stone of flowering time? Science **296:** 285–289.

Smith, T. F., C. Gaitatzes, K. Saxena and E. J. Neer, 1999 The WD repeat: a common architecture for diverse functions. Trends Biochem. Sci. **24:** 181–185.

Soppe, W. J. J., L. Bentsink and M. Koornneef, 1999 The early-flowering mutant *efs* is involved in the autonomous promotion pathway of *Arabidopsis thaliana*. Development **126:** 4763–4770.

van Nocker, S., M. Muszynski, K. Briggs and R. M. Amasino, 2000 Characterization of a gene from *Zea mays* related to the Arabidopsis flowering-time gene *LUMINIDEPENDENS*. Plant Mol. Biol. **44:** 107–122.

Wellensiek, S. J., 1962 Dividing cells as the locus for vernalization. Nature **195:** 307–308.

Wellensiek, S. J., 1964 Dividing cells as the prerequisite for vernalization. Plant Physiol. **39:** 832–835.

Zhang, H., and S. van Nocker, 2002 The *VERNALIZATION INDEPENDENCE 4* gene encodes a novel regulator of *FLOWERING LOCUS C*. Plant J. **31:** 663–667.

Communicating editor: C. S. Gasser