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ARABIDOPSIS *VERNALIZATION INDEPENDENCE* GENES
ARE REQUIRED FOR *FLOWERING LOCUS C* ACTIVATION

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Major Professor's Signature

12/15/04

Date

ARABIDOPSIS *VERNALIZATION INDEPENDENCE* GENES ARE INVOLVED IN *FLOWERING LOCUS C* ACTIVATION

By

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ABSTRACT

ARABIDOPSIS *VERNALIZATION INDEPENDENCE* GENES ARE INVOLVED IN *FLOWERING LOCUS C* ACTIVATION

By

Hua Zhang

Vernalization is the phenomenon of flowering being promoted by long period of near-freezing temperatures (*e.g.*, natural winter). In Arabidopsis, a flowering repressor gene, *FLOWERING LOCUS C* (*FLC*), is believed to be the main target of vernalization. Although several genes have been identified as either positive or negative *FLC* regulators, none of them is required for the vernalization mechanism.

In order to achieve a better understanding of vernalization at the molecular level, a genetic approach has been carried out to identify Arabidopsis genes that promote *FLC* expression. Seven genetic loci, designated *VERNALIZATION INDEPENDENCE* (*VIP*), were identified. All of the *vip* mutants show an early flowering phenotype and suppressed *FLC* mRNA expression. Besides flowering early, the *vip* mutants also show mild developmental pleiotropy, suggesting that VIPs play multiple roles in plant development.

Two of the *VIP* genes, *VIP3* and *VIP4*, have been cloned from the presented dissertation. *VIP3* encodes a protein composed of almost entirely WD repeats, suggesting a role in protein-protein interaction. *VIP4* encodes a protein with sequence homology to a yeast protein Leo1p, a subunit of the yeast transcription complex named Paf1/RNA polymerase II complex (Paf1C). Later research of this laboratory revealed that the VIP2, VIP5 and VIP6 all encode proteins similar to the components of Paf1C.

The sequence homology between these VIPs and Paf1C components suggests that some VIPs, if not all, could be subunits of a plant transcription complex that is similar to the yeast Paf1C.

A genetic approach and a biochemical approach were adopted to test the hypothesis that VIPs may present in a protein complex. My results showed that genetic combinations of *vip4* mutation with mutations in the other six *VIP* genes did not give an enhanced mutant phenotype when compared with either of the parental single mutant. Furthermore, VIP3, VIP4 and VIP6 proteins were shown to physically interact with each other *in vivo*. Both results indicate that VIPs are components of a protein complex.

The outcomes of the presented study suggest that VIPs might represent a previously un-recognized transcriptional regulation mechanism in plants.

Dedicated to my wife Zehua Fu for her eternal love

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ABBREVIATIONS

Chemicals:

Bis-Tris Propane	bis[tris(hydroxymethyl)methylamino]propane
DTE	dithioerythritol
EDTA	ethylenediaminetetraacetic acid
MOPS	3-[N-morpholino]propanesulfonic acid
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate

Genes:

ABH1	*ABA HYPERSENSITIVE 1*
AGL20	*AGAMOUS-LIKE20*
CO	*CONSTANS*
CRY2	*CRYPTOCHROME2*
ELF7	*EARLY FLOWERING 7*
ESD4	*EARLY IN SHORT DAYS4*
FLC	*FLOWERING LOCUS C*
FLD	*FLOWERING LOCUS D*
FLK	*FLOWERING LATE WITH KH MOTIFS*
FRI	*FRIGIDA*
FRL1	*FRIGIDA-LIKE1*

FRL2	*FRIGIDA-LIKE2*
FT	*FLOWERING LOCUS T*
GI	*GIGANTEA*
LD	*LUMINIDEPENDENS*
PHYA	*PHYTOCHROME A*
PIE1	*PHOTOPERIOD INSENSITIVE1*
SOC1	*SUPPRESSOR OF CONSTANS1*
VIN3	*VERNALIZATION INSENSITIVE3*
VIP3	*VERNALIZATION INDEPENDENCE3*
VIP4	*VERNALIZATION INDEPENDENCE4*
VIP5	*VERNALIZATION INDEPENDENCE5*
VIP6	*VERNALIZATION INDEPENDENCE6*
VRN1	*VERNALIZATION1*
VRN2	*VERNALIZATION2*

Chapter 1

Literature review

Unlike animals, higher plants produce their organs post-embryonically (i.e., after germination). This developmental scheme is sustained by the meristem tissue, comprised of a mass of stem cells. The meristem provides cells not only for differentiating into new organs but also for self-renewal. After undergoing a certain period of vegetative growth, a plant acquires the capability of reproductive growth (flowering), a physiological state called competency. Subsequently, the plant will flower once favorable environmental conditions permit. During this phase change, the meristem fate is changed from a vegetative state to a reproductive state, producing cells for flowers and gametophytes. For most of the higher plants, flowering to set seeds is the only way of propagating. Thus, the success of reproductive growth is critical for species continuity.

Most of our knowledge concerning flowering is obtained from studies of the model plant *Arabidopsis thaliana*. A number of genes (Table 1) have been identified to 'time' this phase change. These genes, called flowering-time genes, compose different flowering pathways that sense both endogenous and exogenous cues, ensuring flowering to occur under favorable conditions for seed production (Figure 1). Conceptually, signals from the flowering regulatory pathways are integrated by a limited number of flowering pathway integrator genes, and the activity of the flowering pathway integrators ('processed signals') specifies the expression state of a group of meristem identity genes, which, in turn, control genes determining floral organ identity and patterning.

Table 1. List of Arabidopsis flowering time genes that are discussed in this review.

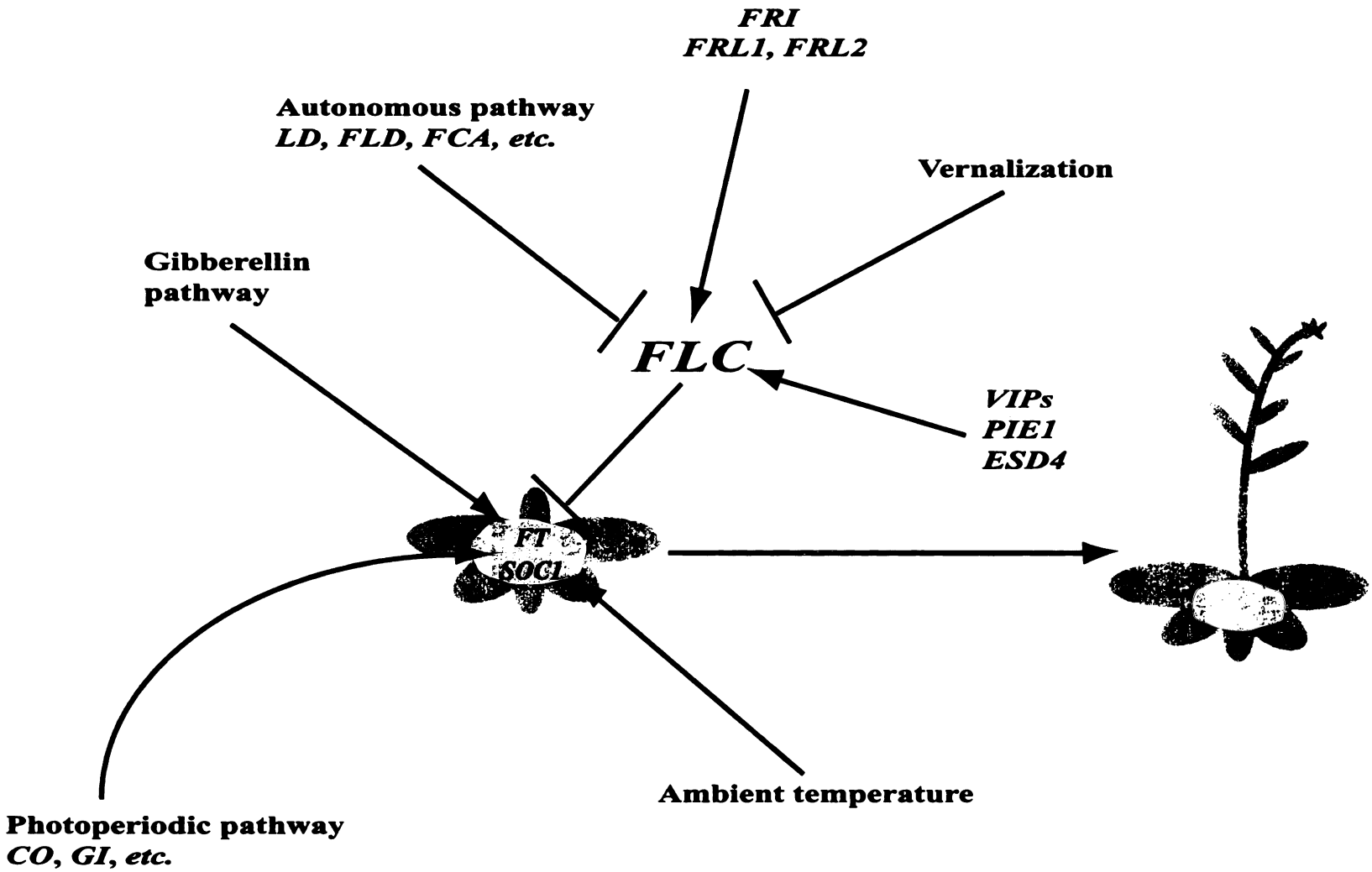
Gene name	Locus	Protein function	Reference
AGL20 (AGAMOUS-LIKE20, a.k.a. SOC1)	At2g45660	MADS-box transcription factor	Lee et al., 2000
CO (CONSTANS)	At5g15840	B-box transcription factor	Putterill et al., 1995
CRY2 (CRYPTOCHROME2)	At1g04400	Blue light receptor	Blazquez et al., 2003
ELF7 (EARLY FLOWERING 7, a.k.a. VIP2)	At1g79730	Protein with homology to the yeast transcriptional activator Paf1p	He et al., 2004; Ek-Ramos and van Nocker, unpublished
ESD4 (EARLY IN SHORT DAYS4)	At4g15880	Nuclear protease regulating	Murtas et al., 2003
FCA	At4g16280	RNA-binding protein	Macknight et al., 1997
FLC (FLOWERING LOCUS C)	At5g10140	MADS-box transcription factor	Michaels and Amasino, 1999; Sheldon et al., 1999
FLD (FLOWERING LOCUS D)	At3g10390	HDAC-associated protein	He et al., 2003
FLK (FLOWERING LATE WITH KH MOTIFS)	At3g04610	RNA-binding protein	Lim et al., 2004
FPA	At2g43410	RNA-binding protein	Schomburg et al., 2001
FRI (FRIGIDA)	At4g00650	Protein with Coiled-coil domains	Johanson et al., 2000
FRL1 (FRIGIDA-LIKE1)	At5g16320	Protein related to FRIGIDA	Michaels et al., 2004
FRL2 (FRIGIDA-LIKE2)	At1g31840	Protein related to FRIGIDA	Michaels et al., 2004
FT (FLOWERING LOCUS T)	At1g65480	Putative kinase inhibitor	Kardailsky et al., 1999; Kobayashi et al., 1999
FVE	At2g19520	Encodes WD-40 protein with metal ion binding ability, similar to yeast MULTICOPY SUPPRESSOR OF IRA1 4	Ausin et al., 2004
FY	At5g13480	Polyadenylation factor	Simpson et al., 2003

Table 1 (cont'd)

Gene name	Locus	Protein function	Reference
GI (*GIGANTEA*)	At1g22770	Protein containing six trans-membrane domains	Fowler et al., 1999; Park et al., 1999
LD (*LUMINIDEPENDENS*)	At4g02560	Homeodomain protein	Lee et al., 1994a
PHYA (*PHYTOCHROME A*)	At1g09570	Red light receptor	Blazquez et al., 2003
PIE1 (*PHOTOPERIOD INSENSITIVE1*)	At3g12810	SWI/SNF-helicase-like protein	Noh and Amasino, 2003
SOC1 (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1, a.k.a. AGL20*)	At2g45660	MADS-box transcription factor	Samach et al., 2000
VIN3 (*VERNALIZATION INSENSITIVE3*)	At5g57380	Protein with fibronectin repeats and PHD domain	Sung and Amasino, 2004
VIP3 (*VERNALIZATION INDEPENDENCE3*)	At4g29830	WD-repeats protein	Zhang et al., 2003
VIP4 (*VERNALIZATION INDEPENDENCE4*)	At5g61150	Protein with homology to the yeast transcriptional activator Leo1p	Zhang and van Nocker, 2002
VIP5 (*VERNALIZATION INDEPENDENCE5*)	At1g61040	Protein with homology to the yeast transcriptional activator Rtf1p	Oh et al., 2004
VIP6 (*VERNALIZATION INDEPENDENCE6, a.k.a. ELF8*)	At2g06210	Protein with homology to the yeast transcriptional activator Ctr9p	Oh et al., 2004; He et al., 2004
VRN1 (*VERNALIZATION1*)	At3g18990	B3 domain DNA-binding protein	Levy et al., 2002
VRN2 (*VERNALIZATION2*)	At4g16845	Su(z)12-like polycomb protein	Gendall et al., 2001

Figure 1. Flowering time control in Arabidopsis. Multiple mechanisms co-exist to give a delicate control of flowering time. The vernalization pathway responds to extended near-freezing temperatures; the autonomous pathway responds to endogenous signals; the photoperiodic pathway responds to changes of day-length, the gibberellin pathway is regulated by phytohormone gibberellin; and the ambient temperature pathway promotes flowering at higher ambient temperatures. This flowering model indicates that these pathways regulate, directly or indirectly, a limited number of flowering pathway integrator genes (*SOC1*, *FT*), which, in turn, controls the expression of the floral meristem identity genes to promote flowering.

In the diagram, the arrow indicates a promotive effect, while the "⊥" indicates an inhibitory effect.


FRI
FRL1, FRL2
Autonomous pathway
LD, FLD, FCA, etc.
Vernalization
Gibberellin
pathway
FLC
VIPs
PIE1
ESD4
FT
SOC1
Ambient temperature
Photoperiodic pathway
CO, GI, etc.

TEMPERATURE AND FLOWERING

As mentioned above, flowering is under the control of both endogenous and environmental cues. One of the environmental factors that affect flowering is temperature, including both ambient and near-freezing temperatures. For some species, high ambient temperatures have a flowering-promotive effect. For example, wild-type Arabidopsis plants flower earlier at 23 °C than at 16 °C (Blazquez et al., 2003). The molecular basis of ambient temperature in flowering-time control was studied recently, and it was found that this mechanism is probably mediated through the blue light receptor CRYPTOCHROME2 (CRY2) and the red light receptor PHYTOCHROME A (PHYA). As Blazquez and his colleague reported, the late-flowering phenotype of *cry2* mutant is exacerbated at 16 °C. The *phy*A mutant flowers slightly late at 23 °C. However, the *cry2* *phy*A double mutant growing at 23 °C flowers as late as the *cry2* single mutant growing at 16 °C (i.e., a more than additive effect was observed) (Blazquez et al., 2003). This observation suggests that the promotive effect of high ambient temperatures is mediated redundantly by photoreceptors CRY2 and PHYA.

For certain plants, flowering can also be accelerated or triggered by a long period of near-freezing temperatures, a phenomenon known as vernalization. It needs to be clarified that vernalization specifically refers to the flowering-promotive effect of chilling treatment, rather than the breakage of dormancy by cold (Lang, 1965). An understanding of vernalization has great practical and fundamental value. Practically, some crops (*e.g.*, winter wheat) need vernalizing cold to flower, and thus the production of these crops is

limited to areas with appreciable cold during winter. An understanding of the mechanism of vernalization will help to develop ways to bypass the requirement for cold, and thus extend the production area of these crops. Fundamentally, vernalization is a developmental process that involves an epigenetic mechanism (see below). In animals, epigenetic mechanisms play key roles in development, by maintaining the expression states of developmental regulatory genes. However, the involvement of epigenetic mechanisms in plant development is not well studied. Thus, an understanding of vernalization will enrich our knowledge of epigenetic gene regulation in development.

THE PHYSIOLOGY OF VERNALIZATION

The physiology of vernalization has been extensively studied. For most species that have been studied, the range of effective chilling temperature for vernalization is typically 1-7 °C (Lang, 1965). Results from localized cold treatment and grafting experiments suggested that, in intact plants, vernalization and the perception of the cold signal for vernalization take place in the shoot apex. In such experiments, it was found that chilling of the shoot apex while maintaining other portions of the plant at a non-inductive temperature was sufficient to promote flowering in celery, beets and chrysanthemums (Curtis and Chang, 1930; Chroboczek, 1934; Schwabe, 1954); and grafting of shoot tips from vernalized *Althaea rosea* onto non-vernalized stock resulted in flowering induction (reviewed in Lang, 1965). The dose-effect curve of cold treatment indicates that vernalization is a quantitative effect, and the duration of chilling to achieve

maximum flowering promotion is species-dependent (Lang, 1965). At least for some species (*e.g.*, sugarbeets and Arabidopsis), the vernalized state can be annulled by several days of high temperatures (*e.g.*, 30 °C) immediately following the cold treatment, a phenomenon known as devernalization (Lang, 1965).

VERNALIZATION AND GIBBERELLIN

It has long been hypothesized that the phytohormone gibberellin (GA) is involved in vernalization. Exogenous application of GA to the biennial plant *Hyoscyamus niger* is capable of flowering induction under long-day conditions in the absence of cold (Lang, 1957). Later, the flowering-inductive effect of GA application on a number of other cold requiring species was also observed (Zeevaart, 1983). Furthermore, in radish, the endogenous GA level was found to increase during the course of a vernalizing cold treatment (Suge, 1970). All of these observations suggest that GA may play a role in vernalization. Nevertheless, recent studies indicate that, at least in Arabidopsis, GA probably is not required for this process. In one of such studies, exogenous GA application did not affect the expression of the *FLOWERING LOCUS C* gene, the main target of vernalization in Arabidopsis (see below) (Sheldon et al., 1999). In another experiment, the endogenous GA level of vernalization-responsive Arabidopsis strains was genetically reduced by a mutation in the *GA1* gene, which encodes an enzyme that catalyes the first step of GA biosynthesis (Sun et al., 1992). Although this *ga1* mutation

can cause a very severe GA deficiency (Sun et al., 1992), it did not cause a loss of vernalization response (Michaels and Amasino, 1999a; Chandler *et al.*, 2000).

THE EPIGENETIC NATURE OF VERNALIZATION

In most cases, plants do not flower immediately after being vernalized, suggesting that plants can somehow 'remember' their past experience of the vernalizing cold. This type of 'plant memory' was excellently demonstrated using *Hyocyamus niger* (Lang, 1965). For flowering, *H. niger* obligately requires both vernalizing cold and long-day conditions. Vernalized *H. niger* could be maintained at the vegetative stage under short-day conditions (non-inductive photoperiods) for up to ~ 300 days. The plant was subsequently able to flower after being provided with inductive, long-day photoperiods (Lang, 1965).

The epigenetic memory of vernalization seems to require mitotic activity, as suggested by regeneration experiments using vernalized *Lunaria biennis*, a biennial (Wellensiek, 1962; 1964). In such experiments, mature leaves and young leaves were marked at the beginning of the cold treatment. After a vernalizing cold treatment, these leaves were detached and regenerated into plants. It was found that plants regenerated from mature leaves remained vegetative, whereas plants regenerated from young leaves could flower without being vernalized. These plant regeneration experiments suggested that the vernalization state is aquired and transmitted mitotically. However, the

vernalization state cannot be transmitted through meiosis (i.e., the progeny of vernalized plants must be re-subjected to cold treatment for flowering).

VERNALIZATION IN ARABIDOPSIS OPERATES PREDOMINATELY VIA SILENCING OF *FLC*

Recently, researchers started to unravel the molecular basis of vernalization, mostly using Arabidopsis as a model. Two types of growth habits, annuals and winter-annuals, exist in this reference plant species. Whereas the commonly used 'lab strains' behave as annuals, flowering soon after germination, most natural ecotypes are winter-annuals, germinating in the fall, remaining vegetative during the winter, and flowering in the following spring. By contrast with the biennials, which have a qualitative requirement for cold treatment for flowering, winter-annuals have a quantitative requirement. Genetic and molecular studies indicate that this naturally occurring flowering-time variation is mainly determined by allelic variations in two genes, namely *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). The functional, dominant *FRI* and semidominant *FLC* act synergistically to confer a winter-annual growth habit, and mutations in either gene create an annual habit (Burn et al., 1993a; Lee et al., 1993, 1994b; Koornneef et al., 1994; Johanson et al., 2000; Michaels et al., 2003).

In Arabidopsis, vernalization is mediated predominantly through silencing of *FLC* (Michaels and Amasino, 1999b; Sheldon *et al.*, 1999). *FLC* encodes a MADS-box transcription factor and acts as a flowering repressor, by suppressing the flowering

pathway integrator genes *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) [*a.k.a. AGAMOUS-LIKE 20* (*AGL20*)] and *FLOWERING LOCUS T* (*FT*), which trigger the floral transition (Michaels and Amasino, 1999b; Sheldon *et al.*, 1999; Lee et al., 2000; Samach et al., 2000; Michaels and Amasino, 2001). After vernalizing cold, *FLC* mRNA abundance decreased to an undetectable level in Arabidopsis strains showing a winter-annual growth habit (Michaels and Amasino, 1999b; Sheldon *et al.*, 1999). *FLC* was found to be highly expressed in shoot and root tips (Michaels and Amasino, 2000). As suggested by the regeneration experiments mentioned above, mitotically active tissues are responsive to vernalization; the spatial expression pattern of *FLC* is consistent with the proposed role of *FLC* being the main target of vernalization.

As mentioned above, vernalization was proposed to involve an epigenetic mechanism. This is also reflected by the kinetics of *FLC* expression. Once down-regulated by cold, the suppressed status of *FLC* is maintained throughout the rest of the plant's life cycle. However, the expression of *FLC* is reset to a high level in the next generation (Michaels and Amasino, 1999b; Sheldon et al., 1999).

It needs to be stressed that vernalization does not operate exclusively through *FLC*. Recently, an *FLC*-independent vernalization mechanism was also proposed, based on the observation that the *flc* null mutant maintains a small vernalization response (Michaels and Amasino, 2001). *FLC* belongs to a six-member MADS-box gene family named *FLC/MADS AFFECTING FLOWERING* (*MAF*). The other five members of this family are *MAF1* [*a.k.a. FLOWERING LOCUS M* (*FLM*) (Scortecci et al., 2001)] -

MAF5. All *MAF*s give a late-flowering phenotype when overexpressed (Ratcliffe et al., 2001; 2003), suggesting that these MAFs, like FLC, act as floral repressors. Although proposed to have evolved from a common ancestor, at least some members of the *FLC/MAF* gene family appear to be involved in different flowering regulatory mechanisms. For example, mutations in *FLC* suppress the late-flowering phenotype of the autonomous pathway mutants (see below); whereas mutations in *MAF1/FLM* suppress the late-flowering phenotype caused by mutations in genes that promote flowering through a photoperiodic response (Scortecci et al., 2001). Like *FLC*, the *MAF*s are subject to repression by vernalizing cold, although to different extent, suggesting that MAFs may act to 'fine-tune' the vernalization response under different environmental conditions (Ratcliffe et al., 2001; 2003; van Nocker, unpublished).

COLD SIGNALING PATHWAYs ASSOCIATED WITH VERNALIZATION MIGHT BE DISTINCT FROM THAT OF COLD ACCLIMATION

The signaling pathway leading to vernalization-associated *FLC* repression is largely unknown. It is possible that vernalization may share some components with other cold response signaling pathways. Two other plant responses that are also observed under vernalization-effective temperatures are cold acclimation and breakage of seed dormancy. Cold acclimation has been extensively studied at the molecular level (Thomashow, 1999).

Cold acclimation is a phenomenon whereby plants aquire the ability to withstanding freezing temperature after being exposed to near-freezing temperatures for a certain period of time (Thomashow, 1999). Plants show pronounced changes during/after the acclimation process, including changes in membrane lipid composition, accumulation of cryoprotectants, and large changes in gene expression (see below) (Thomashow, 1999).

The best known signaling pathway leading to cold acclimation is mediated through the *C-repeat/DRE Binding Factor* (*CBF*) regulon. In the current model of cold acclimation involving this regulon, low temperature activates an ubiquitously present INDUCER OF CBF EXPRESSION protein to induce the expression of *CBF*s. The CBFs, in turn, induce the expression of the whole *CBF* regulon, leading to an increase in cold tolerance (Thomashow, 2001). A negative regulator of *CBF*, designated *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1* (*HOS1*), has been identified from the Arabidopsis ecotype C24, which shows a marginal vernalization response. Interestingly, besides the defects in cold acclimation, the *hos1* mutant was also reported to show an early flowering phenotype and a decrease of *FLC* expression, suggesting a potential role of *HOS1* in vernalization signaling as well (Ishitani et al., 1998; Lee *et al.*, 2001).

Although vernalization and cold acclimation may share some upstream components (e.g., HOS1), it has been suggested that the cold signaling pathway downstream from *CBF* is independent of that leading to *FLC* repression. Overexpression of *CBF1* in Arabidopsis is capable of turning on an array of *Cold Responsive* (*COR*)

genes, resulting in an increase in cold tolerance (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 2002). However, overexpression of *CBF1* in a winter-annual Arabidopsis strain did not cause a decrease in *FLC* expression, nor did it alter the vernalization property of this strain, suggesting that the *CBF1* regulon is not involved in vernalization (Liu *et al.*, 2002).

EPIGENETIC REGULATION OF *FLC* IN VERNALIZATION

Genes that are required for vernalization-associated *FLC* silencing

The establishment and maintenance of epigenetic *FLC* silencing under cold apparently requires the function of the *VERNALIZATION INSENSITIVE3* (*VIN3*) and a set of *VERNALIZATION* (*VRN*) genes (Sung and Amasino, 2004a; Chandler et al., 1996). These genes were identified through a genetic screen for loss of vernalization response. In the *vin3* mutant, *FLC* continues to be expressed at high levels even after vernalizing cold, suggesting that VIN3 is involved in establishing the repression of *FLC* (Sung and Amasino, 2004a). Molecular studies showed that *VIN3* transcripts accumulate during the course of vernalizing cold treatment but become absent again after subsequent growth under warm temperatures (Sung and Amasino, 2004a). The VIN3 protein contains a plant homeodomain (PHD) that is often found in proteins associated with chromatin remodeling complexes (Sung and Amasino, 2004a).

Unlike what was seen in the *vin3* mutant, *FLC* expression decreased to similar levels in *vrn1* or *vrn2* mutant and wild-type plants during cold treatment. However, during the subsequent growth under normal growth temperatures, *FLC* levels increased in

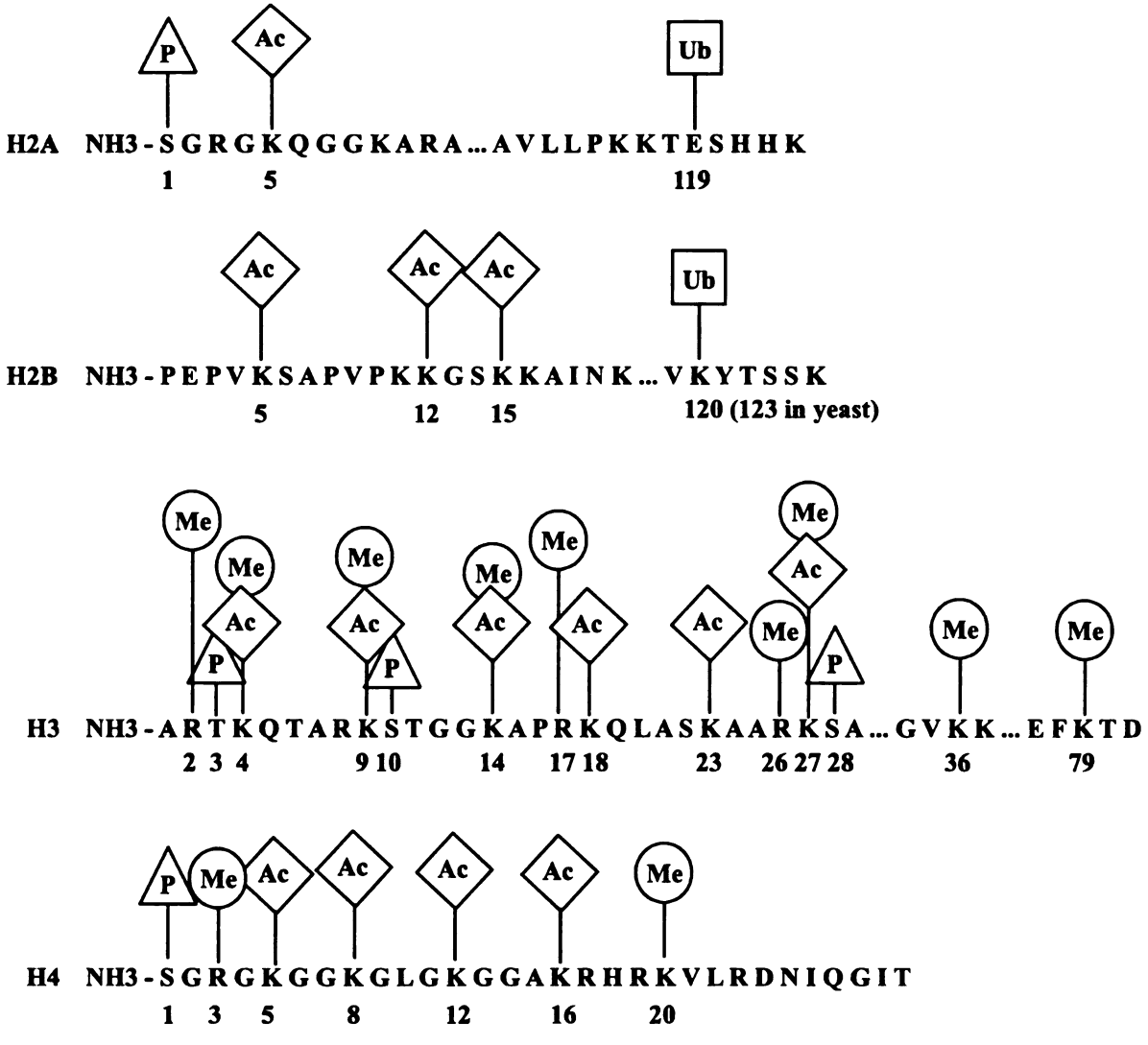
the *vrn1* and *vrn2* mutants, in sharp contrast to the wild type plants, in which *FLC* was maintained at low levels (Gendall *et al.*, 2001; Levy et al., 2003). The *FLC* expression kinetics in *vrn* mutants indicates that VRNs are required to maintain *FLC* in a silenced state, and thus VRNs function downstream of VIN3. *VRN1* encodes a putative DNA binding protein (Levy et al., 2002); *VRN2* encodes a nuclear localized, zinc finger protein, with sequence similarity to the Drosophila Polycomb-group protein Su(Z)12 (Gendall *et al.*, 2001). In Drosophila, Polycomb-group proteins are components of large complexes that reinforce the transcriptionally suppressed state of homeotic genes, probably by packaging and/or maintaining chromatin in states less accessible to transcriptional machinery (Pirrotta, 1997). Consistent with its predicted function, *VRN2* seems to be required to maintain an inaccessible status of the *FLC* gene, as suggested by the observation that, after vernalizing cold, the *FLC* chromatin region is more accessible to the DNase I digestion in a *vrn2* mutant than in wild-type plants (Gendall *et al.*, 2001). Recent findings that Su(Z)12 is a component of a protein complex with histone methyltransferase activity suggest that VRN2 may also function, at least in part, through histone modifications (Kuzmichev et al., 2002; Muller et al., 2002) (see below).

Chromatin histone modification and epigenetic gene regulation

Eukaryotic gene transcription by RNA polymerase II is a highly coordinated process that involves a large number of auxillary factors to facilitate gene recongition and transcription initiation, elongation and termination. In eukaryotes, the genomic DNA

template is packaged into chromatin (and subsequently into chromosomes). The basic repeating unit of chromatin is the nucleosome, which is composed of ~146 bp DNA wound twice around a histone core, composed of an octamer of histone H2A, H2B, H3 and H4. The linker DNA between two adjacent nucleosomes is covered by histone H1. This compact chromatin structure can make the DNA template inaccessible to the transcription machinery. Recent studies on transcription identified a number of transcription factors that modify chromatin structure, by either displacing nucleosomes along the DNA or posttranslationally modifying nucleosomal histones (Svejstrup, 2004). These histone modifications include, but are not limited to, phosphorylation, methylation, acetylation, ubiquitination and sumoylation (Figure 2). Accumulating evidence indicates that posttranslational histone modifications play important roles in epigenetic gene regulation. Potentially, these histone modifications may affect gene transcription by altering higher order chromatin folding, and/or by creating a signal for recruiting additional regulatory elements. In a recently raised 'histone code' hypothesis, the type, number and pattern of the nucleosome histone modifications epigenetically determine the transcriptional state of a certain gene (for reviews, see Jenuwein and Allis, 2001; Fischle et al., 2003).

Figure 2. The types of common nucleosome histone modifications and the potential histone modification sites. P: phosphorylation, AC: acetylation, Ub: ubiquitination, Me: methylation.


P
Ac
Ub
H2A NH3-SGRGKQGGKARA...AVLLPKKTESHHK
1 5 119
Ac
Ac
Ac
Ub
H2B NH3-PEPVKSAPVPKKGSKKAINK...VKYTSSK
5 12 15 120 (123 in yeast)
Me
Me
Ac
P
Me
Ac
P
Me
Ac
Me
Ac
Ac
Me
Ac
P
Me
Me
H3 NH3-ARTKQTARKSTGGKAPRKQLASKAARKSA...GVKK...EFKTD
2 3 4 9 10 14 17 18 23 26 27 28 36 79
P
Me
Ac
Ac
Ac
Ac
Me
H4 NH3-SGRGKGGKGLGKGGAKRHRKVLRDNIQGIT
1 3 5 8 12 16 20

Current model of vernalization-associated *FLC* silencing

Examination of *FLC* chromatin in vernalized wild-type and mutants indicated that there are sequential histone modification events that occur during the vernalizing cold treatment, and such histone modifications depend on the functions of VIN3, VRN1 and VRN2 (Sung and Amasino, 2004a; Bastow et al., 2004). In a recent model of vernalization, VIN3 targets a histone deacetylation (HDAC) mechanism to create a hypoacetylation environment on *FLC* chromatin. Such hypoacetylated chromatin may subsequently recruit a histone methyltransferase (HMT) mechanism(s) that is mediated by VRN1 and VRN2 to methylate histone H3 at lysine-9 (K9) and K27 residues (Sung and Amasino, 2004b). At least in animal systems, H3K9 methylation in euchromatin is required for recruitment of heterochromatin protein1 (HP1) to establish a localized heterochromatic region, associated with long-term gene silencing (Schultz et al., 2002).

DNA methylation and vernalization

More evidence that epigenetic modification of chromatin structure is involved in *FLC* regulation or vernalization comes from the study of Arabidopsis transgenic lines defective in DNA methylation, a process that is tightly linked to chromatin dynamics. Prolonged growth at low temperature results in a hypomethylation in Arabidopsis genome, and a 5-azacytidine treatment, which causes a reduction of methylated cytocine in DNA was capable of promoting flowering on vernalization-responsive Arabidopsis strains, but not on vernalization-nonresponsive lines (Burn et al., 1993b). Genetically

reducing the genomic methylation level via antisense expression of a cytosine methyltransferase gene in a winter-annual Arabidopsis strain has been found to remarkably decrease the *FLC* transcript level in a vernalization-independent manner (Finnegan *et al.*, 1998; Sheldon *et al.*, 1999). However, no change of *FLC* DNA methylation status was detected before and after chilling (J. Finnegan, personal communication).

FLC NEGATIVE REGULATORS – "AUTONOMOUS PATHWAY GENES"

Besides being suppressed by vernalization, *FLC* is also negatively regulated by genes composing a so-called autonomous flowering promotion pathway (Figure 1). The autonomous pathway is represented by seven genes, *FCA*, *FY*, *FVE*, *FPA*, *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS D* (*FLD*), and *FLOWERING LATE WITH KH MOTIFS* (*FLK*), which have been identified through screening for late-flowering mutants in early-flowering Arabidopsis ecotypes. The autonomous pathway mutants flower late in both long-day and short-day photoperiodic conditions. However, these mutants are vernalization responsive (Martinez-Zapater and Somerville, 1990; Koornneef *et al.*, 1991), indicating that the autonomous pathway genes are not required for vernalization. Compared to the wild-type plants, the autonomous pathway mutants express *FLC* mRNA to high levels (Michaels and Amasino, 1999b; Sheldon *et al.*, 1999; Lim et al., 2004). Additional evidence for autonomous pathway genes negatively regulating *FLC* expression comes from the observation that an *flc* null mutation is

epistatic to mutations in autonomous pathway genes *FCA*, *FPA*, *FVE*, or *LD* (Michaels and Amasino, 2001).

Although autonomous pathway mutants behave similarly, it is not likely that autonomous pathway genes are involved in a simple linear pathway. Clear genetic interactions exist when combining some of the autonomous pathway mutants (Koornneef et al., 1998). An additive effect was observed when combining *fca* and *fve*, and a more than additive effect was observed between *fca* and *fpa*, suggesting that *FCA* operates in a mechanism distinct from that of *FPA* and *FVE*. The *fpa* mutant is epistatic to *fve*, which indicates that *FPA* and *FVE* may act in the same mechanism and locates *FVE* downstream of *FPA*. The *fca* mutant showed a less than additive interaction with *fy*, implying that *FCA* and *FY* operate in the same mechanism. The *fpa fy* double mutant might be lethal because no such double mutant was recovered. The fact that the *fpa fca* mutant is viable would place *FCA* upstream to *FY*, because otherwise the *fpa fca* double mutant is expected to be lethal (Koornneef et al., 1998).

All of the autonomous pathway genes mentioned above have been cloned. From analysis of the proteins that they encode, these genes appear to regulate *FLC* through partially distinct mechanisms. *LD* encodes a protein with a homeobox and putative nuclear localization signal and may act as a transcription factor (Lee *et al.*, 1994a). *FCA*, *FPA* and *FLK* encode proteins containing potential RNA-binding domains (Macknight *et al.*, 1997; Schomburg *et al.*, 2001, Lim et al., 2004), and FY, which physically interacts with FCA, is related to the yeast mRNA 3'-end processing factor Pfs2p (Simpson et al.,

2003), indicating that these proteins may have roles in posttranscriptional control of gene expression. FLD is homologous to a human protein, KIAA0601, a component of the histone deacetylase 1,2 (HDAC1/2) complex (He et al., 2003), while FVE is homologous to mammalian retinoblastoma-associated proteins (RbAp) and yeast multicopy suppressor of IRA1 (MSI) (Ausin et al., 2004; Kim et al., 2004). RbAp was shown to interact with the HDAC that is recruited by the transcriptional repressor Retinoblastoma protein (Nicolas et al., 2000). This evidence suggests that FLD and FVE might be involved in a histone deacetylation mechanism. And indeed, histones of the *FLC* chromatin were shown to be hyperacetylated in both *fld* and *fve* mutants (He et al., 2003; Ausin et al., 2004). More significantly, FVE and another Arabidopsis MSI-like protein, AtMSI1, were found to be involved in a polycomb group-like complex and chromatin assembly facter-1 (CAF-1), respectively, suggesting their potential roles in epigenetic gene silencing and chromatin assembly (Kohler et al., 2003; Kaya et al., 2001).

FLC POSITIVE REGULATORS

FRIGIDA and *FRIGIDA-LIKE* genes

In addition to being negatively regulated by autonomous pathway genes and vernalizing cold, *FLC* was also found to be positively regulated by *FRIGIDA* (*FRI*) and *FRI LIKE1* (*FRL1*). Together with another related gene *FRL2*, *FRI* and *FRL1* belong to one clade of a seven-member gene family named *FRL1* (Michaels et al., 2004). Of this gene family, *FRI* has been the best-studied member. This is mainly because the

commonly used Arabidopsis 'lab strains' carry a dysfunctional *FRI* allele or a weak *FLC* allele, and allelic variation at *FRI* and *FLC* are major determinants of the natural variability of flowering habit in Arabidopsis (Johanson et al., 2000; Michaels et al., 2003).

FRI is epistatic to autonomous pathway genes for *FLC* regulation, as shown by the fact that in an Arabidopsis introgression line containing both functional *FRI* and a functional autonomous pathway, *FLC* expression is elevated, and the strain behaves as a winter-annual (i.e., vernalization responsive) (Michaels and Amasino, 1999b; Lee *et al.*, 1994b). *FRI* encodes a protein without homology to any protein with known function. However, the predicted coiled-coil domains in FRI suggest that additional protein partners may be required for FRI function (Johanson *et al.*, 2000). More extensive molecular study of *FRI* has not yet been reported. It must be pointed out that although both *FRI* and the autonomous pathway genes control *FLC* expression, none of them is required for cold-associated *FLC* silencing, because, as mentioned above, the autonomous pathway mutants, lacking both functional *FRI* and autonomous pathway genes, maintain an intact vernalization response.

The activation of *FLC* by FRI requires FRL1. Mutations in FRL1 suppress the late flowering phenotype caused by dominant *FRI* but has no or little effect on the late flowering phenotype caused by mutations in *LD* or *FPA*, suggesting that the function of FRL1 is specific to *FRI* containing background (Michaels et al., 2004). Accordingly, FRL1 is not required for the high *FLC* expression caused by mutations in autonomous pathway genes *LD* or *FPA* (Michaels et al., 2004). FRL1 may share partial functional

redundancy with FRL2, because a *frl1 frl2* double mutant flowers earlier than a *fri1* single mutant (Michaels et al., 2004). However, although FRL1 is homologous to FRI, they do not seemly have redundant functions, because overexpressing of *FRL1* cannot complement the *fri* mutant and *vise versa* (Michaels et al., 2004).

VERNALIZATION INDEPENDENCE genes

The high *FLC* expression level in autonomous pathway mutants (lacking functional FRI) strongly suggests that there are additional *FLC* activators. Identification of these *FLC* positive regulators, which can be accomplished by analyzing early-flowering mutants in a winter-annual background, is a necessary step to better understand the *FLC* regulatory mechanism and, ultimately, vernalization.

Recently, a group of *vernalization independence* (*vip*) mutants have been isolated to define novel *FLC* positive regulators (Zhang and van Nocker, 2002; Zhang *et al.*, 2003). Molecular characterization of *VIP*s suggests that these proteins might be involved in a transcriptional regulation complex related to the yeast Paf1/RNA polymerase II complex (this dissertation).

EARLY IN SHORT DAYS4 and PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1

Four additional *FLC* activators that do not belong to the VIP group have also been reported. These non-VIP *FLC* activators include FRL1, FRL2 (see above), EARLY IN

SHORT DAYS4 (ESD4) and PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 (PIE1). The *frl1* mutant was identified from a genetic screen similar to the one that was used to identify the VIPs (Michaels et al., 2004; Zhang and van Nocker, 2002), conversely, *esd4* and *pie1* mutants, both flowering early under short-day conditions, were isolated from genetic screens that were not designed to target *FLC* regulators (Reeves et al., 2002; Noh and Amasino, 2003).

The ESD4 protein is similar to yeast and animal proteases that are important for SMALL UBIQUITIN-RELATED MODIFIER (SUMO) processing and recycling, and it appears that the main function of the ESD4 in Arabidopsis is SUMO recycling (Murtas et al., 2003). SUMOylation of a protein may regulate its localization, activity or stability (Melchior, 2000; Muller et al., 2001). Intriguingly, SUMOylation was also shown to be a type of histone modification, and possibly a part of the 'histone code' (Shiio and Eisenman, 2003), raising the possibility that ESD4 might also be involved in chromatin dynamics.

Being homologous to the ISWI and SWI2/SNF2 class of proteins, PIE1 is probably involved in modifying chromatin structures (Noh and Amasino, 2003). In Drosophila, some proteins of this family (e.g., brahma and zeste) were characterized as Trithorax group (TrxG) proteins, which keep homeotic genes actively expressed, in an opposite manner to the PcG proteins (Kennison, 1995). Thus, hypothetically, PIE1 may participate in a TrxG like protein complex to activate *FLC* by changing chromatin structures (Noh and Amasino, 2003).

VERNALIZATION IN CROPS

Many varieties of crop plants also show a vernalization response (Table 2), and the molecular basis of vernalization has also been studied in some of these crop plants. It has been suggested that the *FLC*-mediated vernalization mechanism might be conserved within the *Brassica* species. Overexpressing a *Brassica napus FLC* orthologue in Arabidopsis delayed flowering, and vernalizing cold dramatically reduced the level of *BnFLC* transcripts in winter-annual *B. napus* cultivars (Tadege *et al.*, 2001). Furthermore, the *FLC*-activating system involving VIPs might also be conserved, because proteins immunoreactive to VIP3, VIP4 and VIP6 antibodies were found to be present in *Brassica oleracea* (Cauliflower) (Chapter 4, Figure 5).

Although vernalization might act through an *FLC*-like mechanism in *Brassica* species, no clear homologues of *FLC* are present in cereals (Henderson et al., 2003), suggesting that vernalization operates through a different mechanism in cereal plants. Genetic studies indicated that, in wheat, the vernalization response is determined by the *VRN1* and *VRN2* loci. Although bearing the same names as the Arabidopsis *VRN1* and *VRN2*, the wheat *VRN1* and *VRN2* do not have any relationship with the Arabidopsis ones (see below; and unless it is pointed out, the *VRN1* and *VRN2* refer to the wheat *VRN1* and *VRN2* genes/loci in this section). Dominant alleles of *VRN1* confer a spring annual growth habit, whereas dominant alleles of *VRN2* confer a winter-annual growth habit (Dubcovsky et al., 1998). Genetic studies showed that the dominant *VRN1* allele is

Table 2. Major field crops with vernalization response*.

Species	Common name
Allium cepa	Onion
Avena sativa	Oat
Beta vulgaris	Beet
Brassica napus	Rape
Brassica oleracea	Cauliflower
Cicer arietinum	Chickpea
Dactylis glomerata	Cocksfoot
Daucus carota	Carrot
Hordeum vulgare	Barley
Lactuca sativa	Lettuce
Lens culinaris	Lentil
Linum usitatissimum	Flax
Lolium perenne	Ryegrass
Papaver somniferum	Poppy
Pisum sativum	Pea
Raphanus sativus	Radish
Secale cereale	Rye
Spinacia oleracea	Spinach
Trifolium repens	White clover
Triticum aestivum	Wheat
Vicia taba	Faba bean

* This table is a re-edited version of TABLE 1 from Henderson et al., (2003).

epistatic to the dominant *VRN2* allele, the dominant *VRN2* allele is epistatic to the recessive *vrn1* allele, and the recessive *vrn2* allele is epistatic to the recessive *vrn1* allele (Tranquilli and Dubcovsky, 2000). This is consistent with a model whereby *VRN2* acts upstream of *VRN1*.

Both *VRN1* and *VRN2* have recently been identified by a positional cloning approach. *VRN1* encodes a MADS-box protein homologous to the Arabidopsis APETALA1 (AP1) protein, which is involved in determining floral meristem identity (Yan et al., 2003; Liljegren et al., 1999). In wheat cultivars with a winter-annual growth habit, the *VRN1* transcripts were not detectable until after vernalizing cold; while in an accession that does not require vernalization, *VRN1* was expressed independently of cold treatment (Yan et al., 2003). Sequence analysis showed that in spring accessions, there is a 20bp deletion in the *VRN1* promoter region, presumably abrogating the binding of a transacting repressor (Yan et al., 2003; 2004).

Expression profiling showed that *VRN2* mRNA was down-regulated by vernalizing cold, with a kinetics opposite to that of *VRN1* (Yan et al., 2004), suggesting that VRN2 may act as a flowering repressor. *VRN2* encodes a protein similar to Arabidopsis CONSTANS (CO) and CO-like proteins (Yan et al., 2004). These Arabidopsis proteins, all containing a CO, CO-like, and TOC1 (CCT) domain, were shown to be involved in a flowering mechanism responding to inductive photoperiod (Putterill et al., 1995). The CCT domain was proposed to mediate protein-protein interactions (Kurup et al., 2000). A survey of a large collection of wheat accessions with

winter or spring growth habit suggested that, besides the deletion in the *VRN1* promoter, mutation in the *VRN2* CCT domain or a complete deletion of the *VRN2* gene from the genome are other determinants for the spring growth habit in wheat (Yan et al., 2004).

CONCLUSIONS

The identification of *FLC* created a boost for understanding the molecular basis of vernalization. More and more results support the hypothesis that, in Arabidopsis, a mechanism involving histone modifications participates in the epigenetic silencing of *FLC* during vernalization. There are still many questions that need to be answered. What is the upstream receptor that perceives the vernalizing cold signal? Is this receptor common for other cold related processes, for example, acclimation? What are the factor(s), if any, that operate between *FLC* and its activators or repressors? How are the *FLC* activation mechanisms counteracted by vernalization? How is the epigenetic silencing of *FLC* reset for the next generation? As seen above, results from wheat studies suggest that plants have "chosen" different proteins to regulate the vernalization response; is the epigenetic silencing system in wheat different from that seen in Arabidopsis? Continuing work on these two representative systems will hopefully answer these questions.

References

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004) Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat. Genet. 36: 162-166.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., and Dean, C. (2004) Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature 427: 164-167.

Bezerra, I.C., Michaels, S.D., Schomburg, F.M., and Amasino, R.M. (2004) Lesions in the mRNA cap-binding gene *ABA HYPERSENSITIVE1* suppress *FRIGIDA*-mediated delayed flowering in Arabidopsis. Plant J. 40: 112-119.

Blazquez, M.A., Ahn, J.H., and Weigel, D. (2003) A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. Nat. Genet. 33: 168-171.

Burn, J.E., Smyth, D.R., Peacock, W.J., and Dennis ES. (1993a) Genes conferring late flowering in *Arabidopsis thaliana*. Genetica 90: 145-157.

Burn, J.E., Bagnall, D.J., Metzger, J.D., Dennis, E.S., and Peacock, W.J. (1993b) DNA methylation, vernalization, and the initiation of flowering. Proc. Natl. Acad. Sci. USA 90: 287-291.

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

Chandler, J., Martinez-Zapater, J.M., and Dean, C. (2000) Mutations causing defects in the biosynthesis and response to gibberellins, abscisic acid and phytochrome B do not inhibit vernalization in Arabidopsis *fca-1*. Planta 210: 677-682.

Chroboczek, E. (1934) Study of some ecological factors influencing seed-stalk development in beets (Beta vulgaris). Cornell Agric. Exp. Stat. Mem. No. 154.

Curtis, O.F. and Chang, H.T. (1930) The relative effectiveness of the temperature of the crown as contrasted with that of the rest of the plant upon the flowering of celery plants. Amer. J. Bot. 17: 1047-1048.

Dubcovsky, J., Lijavetzky, D., Appendino, L., and Tranquilli, G. (1998) Comparative RFLP mapping of Triticum monococcum genes controlling vernalization requirement. Theor. Appl. Genet. 97: 968-975.

Finnegan, E.J., Genger, R.G., Kovac, K., Peacock, W.J., and Dennis, E.S. (1998) Methylation controls the low-temperature induction of flowering in Arabidopsis. Proc. Natl. Acad. Sci. USA 95: 5824-5829.

Fischle, W., Wang, Y., and Allis, C.D. (2003) Binary switches and modification cassettes in histone biology and beyond. Nature 425: 475-479.

Gendall, A.R., Levy, Y.Y, Wilson, A., and Dean, C. (2001) The *VERNALIZATION2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell 107: 525-535.

He, Y., Michaels, S., and Amasino, R. (2003) Regulation of flowering time by histone acetylation in Arabidopsis. Science 302: 1751-1754.

Henderson, I.R., Shindo, C., and Dean, C. (2003) The need for winter in the switch to flowering. Annu. Rev. Genet. 37: 371-392.

Ishitani, M., Xiong, L., Lee, H., Stevenson, B., and Zhu, J.K. (1998) *HOS1*, a genetic locus involved in cold-responsive gene expression in arabidopsis. Plant Cell 10: 1151-1161.

Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O., and Thomashow M. F. (1998) Arabidopsis *CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science 280: 104-106.

Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. Science 293: 1074-1080.

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

Kaya, H., Shibahara, K., Taoka, K., Iwabuchi, M., Stillman, B., and Araki, T. (2001) *FASCIATA* genes for chromatin assembly factor-1 in Arabidopsis maintain the cellular organization of apical meristems. Cell 104: 131-142.

Kim, H.J., Hyun, Y., Park, J.Y., Park, M.J., Park, M.K., Kim, M.D., Kim, H.J., Lee, M.H., Moon, J., Lee, I., and Kim, J. (2004) A genetic link between cold responses and flowering time through FVE in Arabidopsis thaliana. Nat. Genet. 36: 167-171.

Kohler, C., Hennig, L., Bouveret, R., Gheyselinck, J., Grossniklaus, U., and Gruissem, W. (2003) Arabidopsis MSI1 is a component of the MEA/FIE polycomb group complex and required for seed development. EMBO J. 22: 4804-4814.

Koornneef, M., Hanhart, C.J., and Van Der Veen, J.H. (1991) A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana. Mol. Gen. Genet. 229: 57-66.

Koornneef, M., Blankestijn-de Vries, H., Hanhart, C., Soppe,W., and Peeters, T. (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. Plant J. 6: 911-919.

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

Kurup, S., Jones, H.D., and Holdsworth, M.J. (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing Arabidopsis seeds. Plant J. 21: 143-155.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. 16: 2893-2905.

Lang, A. (1957) The effect of gibberellin on flower formation. Proc. Natl. Acad. Sci. U.S.A. 43: 709-717.

Lang, A. (1965) Physiology of flower initiation. In Encyclopedia of Plant Physiology (ed. W. Ruhland), pp. 1371-1536. Springer-Verlag, Berlin.

Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M., and Lee, I. (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev. 14: 2366-2376.

Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B., and Zhu, J-K (2001) The Arabidopsis *HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. Genes Dev. 15(7): 912-924.

Lee, I., Bleecker, A., and Amasino, R.M. (1993) Analysis of naturally occurring late flowering in *Arabidopsis thaliana*. Mol. General Genet. 237: 171-176.

Lee, I., Aukerman, M.J., Gore, S.L., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C., Feldmann, K.A., and Amasino, R.M. (1994a) Isolation of *LUMINIDEPENDENS*: A gene involved in the control of flowering time in Arabidopsis. Plant Cell 6: 75-83.

Lee, I., Michaels D.D., Masshardt A.S., and Amasino, R.M. (1994b) 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., Mesnage, S., Mylne, J.S., Gendall, A.R. and Dean, C. (2002) Multiple Roles of Arabidopsis VRN1 in Vernalization and Flowering Time Control. Science 297: 243-246.

Liljegren SJ, Gustafson-Brown C, Pinyopich A, Ditta GS, Yanofsky MF. (1999) Interactions among APETALA1, LEAFY, and TERMINALFLOWER1 specify meristem fate. Plant Cell 11: 1007-1018.

Lim, M.H., Kim, J., Kim, Y.S., Chung, K.S., Seo, Y.H., Lee, I., Kim, J., Hong, C.B., Kim, H.J., and Park, C.M. (2004) A new Arabidopsis gene, *FLK*, encodes an RNA binding protein with K homology motifs and regulates flowering time via FLOWERING LOCUS C. Plant Cell 16: 731-740.

Liu, J., Gilmour, S.J., Thomashow, M.E, and van Nocker, S. (2002) Cold signalling associated with vernalization in Arabidopsis thaliana does not involve CBF1 or abscisic acid. Physiol. Plant. 114: 125-134.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997) *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding motifs. Cell 89: 737-745.

Martinez-Zapater, J.M. and Somerville, C.R. (1990) Effect of light quality and vernalization on late-flowering mutants of Arabidopsis thaliana. Plant Physiol. 92: 770-776.

Melchior, F. (2000). SUMO: Nonclassical ubiquitin. Annu. Rev. Cell Dev. Biol. 16: 591-626.

Michaels, S.D. and Amasino, R.M. (1999a) The gibberellic acid biosynthesis mutant *ga1-3* of *Arabidopsis thaliana* is responsive to vernalization. Dev. Genet. 25: 194-198.

Michaels, S.D. and Amasino, R.M. (1999b) *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 Amasino, R.M. (2000) Memories of winter: vernalization and the competence to flower. Plant Cell Environ. 23: 1145-1153.

Michaels, S.D. and Amasino, R.M. (2001) Loss of FLOWERING LOCUS C Activity Eliminates the Late-Flowering Phenotype of *FRIGIDA* and Autonomous Pathway Mutations but Not Responsiveness to Vernalization. The Plant Cell 13: 935-941.

Michaels S.D., He, Y., Scortecci, K.C., Amasino, R.M. (2003) Attenuation of *FLOWERING LOCUS C* activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. Proc Natl Acad Sci U.S.A. 100: 10102-10107.

Michaels, S.D., Bezerra, I.C., and Amasino, R.M. (2004) *FRIGIDA*-related genes are required for the winter-annual habit in Arabidopsis. Proc Natl Acad Sci U.S.A. 101: 3281-3285.

Mueller, C.L. and Jaehning, J.A. (2002) Ctr9, Rtf1, and Leo1 Are Components of the Paf1/RNA Polymerase II Complex. Mol. Cell. Biol. 22: 1971-1980.

Muller, S., Hoege, C., Pyrowolakis, G., and Jentsch, S. (2001). SUMO, ubiquitin's mysterious cousin. Nat. Rev. Mol. Cell Biol. 2: 202-210.

Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111: 197-208.

Murtas, G., Reeves, P.H., Fu, Y.F., Bancroft, I., Dean, C., and Coupland, G. (2003) A nuclear protease required for flowering-time regulation in Arabidopsis reduces the abundance of SMALL UBIQUITIN-RELATED MODIFIER conjugates. Plant Cell 15: 2308-2319.

Ng, H.H., Dole, S., and Struhl, K. (2003a). The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. J. Biol. Chem. 278: 33625-33628.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2003b). Targeted recruitment of Set1 histone methylase by elongating PolII provides a localized mark and memory of recent transcriptional activity. Mol. Cell 11: 709-719.

Nicolas, E., Morales, V., Magnaghi-Jaulin, L., Harel-Bellan, A., Richard-Foy, H., and Trouche, D. (2000) RbAp48 belongs to the histone deacetylase complex that associates with the retinoblastoma protein. J Biol Chem. 275: 9797-9804.

Noh, Y.S., and Amasino, R.M. (2003). *PIE1*, an ISWI family gene, is required for *FLC* activation and floral repression in Arabidopsis. Plant Cell 15: 1671-1682.

Oh, S., Zhang, H., Ludwig, P., and van Nocker, S. (2004) A mechanism related to the yeast transcriptional regulator Paf1C is required for expression of the Arabidopsis *FLC/MAF* MADS-box gene family. Plant Cell 16: 2940-2953.

Pirrotta, V. (1997) PcG complexes and chromatin silencing. Curr. Opin. Genet. Dev. 7: 249-258.

Putterill, J., Robson, F., Lee, K., Simon, R., and Coupland, G. (1995) The *CONSTANS* gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell 80: 847-858.

Ratcliffe, O.J., Kumimoto, R.W., Wong, B.J., and Riechmann, J.L. (2003). Analysis of the Arabidopsis *MADS AFFECTING FLOWERING* gene family: *MAF2* prevents vernalization by short periods of cold. Plant Cell 15: 1159-1169.

Ratcliffe, O.J., Nadzan, G.C., Reuber, T.L., and Riechmann, J.L. (2001). Regulation of flowering in Arabidopsis by an FLC homologue. Plant Physiol. 126: 122-132.

Reeves, P.H., Murtas, G., Dash, S., and Coupland, G. (2002) *early in short days 4*, a mutation in Arabidopsis that causes early flowering and reduces the mRNA abundance of the floral repressor *FLC*. Development 129: 5349-5361.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science 288: 1613-1616.

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

Schultz, D.C., Ayyanathan, K., Negorev, D., Maul, G.G., and Rauscher, F.J. 3rd. (2002) SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev. 16: 919-932.

Schwabe, W.W. (1954) Factors controlling flowering in the chrysanthemum. IV. The site of vernalization and translocation of the stimulus. J. Exp. Bot. 5: 389-400.

Scortecci KC, Michaels SD, Amasino RM. (2001) Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering. Plant J. 26:229-36.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999) The *FLF* MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell 11: 445-458.

Shiio, Y., and Eisenman, R.N. (2003) Histone sumoylation is associated with transcriptional repression. Proc. Natl. Acad. Sci. U.S.A. 100: 13225-13230.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., and Dean, C. (2003) FY is an RNA 3' end-processing factor that interacts with FCA to control the Arabidopsis floral transition. Cell 113: 777-787.

Suge, H. (1970) Changes of endogenous gibberellins in vernalized radish plants. Plant Cell Physiol. 11: 729-735.

Sun, T.P., Goodman, H.M., and Ausubel, F.M. (1992) Cloning the Arabidopsis *GA1* locus by genomic subtraction. Plant Cell 4: 119-128.

Sung, S., and Amasino, R.M. (2004a) Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature 427: 159-164.

Sung, S., and Amasino, R.M. (2004b) Vernalization and epigenetics: how plants remember winter. Curr. Opin. Plant Biol. 7: 4-10.

Svejstrup, J.Q. (2004) The RNA polymerase II transcription cycle: cycling through chromatin. Biochim. Biophys. Acta. 1677: 64-73.

Tadege, M., Sheldon, C.C., Helliwell, C.A., Stoutjesdijk, P., Dennis, E.S., and Peacock, W.J. (2001) Control of flowering time by *FLC* orthologues in Brassica napus. Plant J. 28: 545-553.

Thomashow, M.F. (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 571-599.

Thomashow, M.F. (2001) So what's new in the field of plant cold acclimation? Lots! Plant Physiol. 125: 89-93.

Tranquilli, G., and Dubcovsky, J. (2000) Epistatic interaction between vernalization genes *Vrn-A^m1* and *Vrn-A^m2* in diploid wheat. J Hered. 91: 304-306.

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.

Yan, L., Loukoianov, A., Blechl, A., Tranquilli, G., Ramakrishna, W., SanMiguel, P., Bennetzen, J.L., Echenique, V., and Dubcovsky, J. (2004) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science 303: 1640-1644.

Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T., and Dubcovsky, J. (2003) Positional cloning of the wheat vernalization gene *VRN1*. Proc. Natl. Acad. Sci. U.S.A. 100: 6263-6268.

Zeevaart, J.A.D. (1983) Gibberellins and flowering. In: Crozier A, editor. The biochemistry and physiology of gibberellins. Vol 2. New York: Praeger. p 333-374.

Zhang, H. and van Nocker, S. (2002) The *VERNALIZATION INDEPENDENCE4* gene encodes a novel regulator of *FLOWERING LOCUS C*. Plant J 31: 663-673.

Zhang, H., Ransom, C., Ludwig, P., and van Nocker, S. (2003) Genetic analysis of early-flowering mutants in Arabidopsis defines a class of pleiotropic developmental regulator required for activity of the flowering-time switch *FLOWERING LOCUS C*. Genetics 164: 347-358.

Chapter 2

The *VERNALIZATION INDEPENDENCE4* gene encodes a novel regulator of *FLOWERING LOCUS C*

This chapter is a re-edited version of my publication **Zhang, H.** and van Nocker, S. (2002) Plant J. 31: 663-673.

Abstract

The late-flowering, vernalization-responsive habit of many *Arabidopsis* ecotypes is mediated predominantly through repression of the floral programme by the *FLOWERING LOCUS C* (*FLC*) gene. To better understand this repressive mechanism, we have taken a genetic approach to identify novel genes that positively regulate *FLC* expression. We identified recessive mutations in a gene designated *VERNALIZATION INDEPENDENCE 4* (*VIP4*), that confer early flowering and loss of *FLC* expression in the absence of cold. We cloned the *VIP4* gene and found that it encodes a highly hydrophilic protein with similarity to proteins from yeasts, *Drosophila*, and *Caenorhabditis elegans*. Consistent with a proposed role as a direct activator of *FLC*, *VIP4* is expressed throughout the plant in a pattern similar to that of *FLC*. However, unlike *FLC*, *VIP4* RNA expression is not down-regulated in vernalized plants, suggesting that *VIP4* is probably not sufficient to activate *FLC*, and that *VIP4* is probably not directly involved in a vernalization mechanism. Epistasis analysis suggests that *VIP4* could act in a separate pathway from previously identified *FLC* regulators, including *FRIGIDA* and the autonomous flowering promotion pathway gene *LUMINIDEPENDENS*. Mutants lacking detectable *VIP4* expression flower earlier than *FLC* null mutants, suggesting that *VIP4* regulates flowering-time genes in addition to *FLC*. Floral morphology is also disrupted in *vip4* mutants; thus, *VIP4* has multiple roles in development.

Introduction

In many plants, flowering is not initiated until after an extended period of growth in the cold. In the natural environment, this mechanism allows flowering and seed production to occur only after winter. In the many ecotypes of *Arabidopsis thaliana* that exhibit this type of flowering habit, repression of flowering is mediated predominantly through the activity of the MADS-box gene *FLOWERING LOCUS C* (*FLC*) (Koornneef *et al.*, 1994; Lee *et al.*, 1994b; Michaels and Amasino, 1999; Sheldon *et al.*, 1999). In current models of flowering, the repressive mechanism involving *FLC* acts antagonistically with promotive pathways associated with GA biosynthesis/sensitivity and perception of inductive photoperiods (Simpson *et al.*, 1999). The accelerated flowering of plants treated with GAs, or grown in inductive photoperiods, is not accompanied by greatly decreased *FLC* RNA expression (Michaels and Amasino, 1999; Sheldon *et al.*, 1999), and *FLC* does not appear to be developmentally regulated (Rouse *et al.*, 2002; Sheldon *et al.*, 1999), indicating that flowering pathways are integrated predominantly 'downstream' of *FLC*. However, both genetic and molecular experiments have suggested that some 'crosstalk' occurs among pathways (Koornneef *et al.*, 1998a; Rouse *et al.*, 2002), and thus these flowering mechanisms cannot be proposed to act completely independently.

FLC is subject to both positive and negative regulation, and several flowering-time genes are known that act as strong, 'upstream' regulators of *FLC*. For example, genes in the so-called autonomous pathway, including *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS D* (*FLD*), *FPA*, *FVE*, *FY*, and *FCA*, act to repress *FLC* (Koornneef

et al., 1991, 1998a). This regulation occurs at least partly at the RNA level, as *FLC* RNA is expressed to high levels in autonomous-pathway mutants relative to wild-type plants (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Several of the autonomous-pathway genes have now been characterized at the molecular level. *LD* encodes a nuclear protein containing a diverged homeodomain and an acidic carboxyl-terminal region enriched in glutamine residues (Aukerman and Amasino, 1996; Lee *et al.*, 1994a; van Nocker *et al.*, 2000) suggesting that it could act as a transcriptional regulator. Two other autonomous-pathway genes, *FCA* and *FPA,* encode proteins containing potential RNA-binding domains (Macknight *et al.*, 1997; Schomburg *et al.*, 2001), suggesting that they function in post-transcriptional control of expression.

The *FRIGIDA* (*FRI*) gene also regulates *FLC* RNA expression, but, in contrast to the autonomous-pathway genes, acts in a promotive manner (Koornneef *et al.*, 1998b; Michaels and Amasino, 1999). The predicted FRI protein does not exhibit strong homology with any other protein of known function, but exhibits coiled-coil domains, suggesting that it interacts with protein partner(s) (Johanson *et al.*, 2000). Although it is now clear that other positive regulators of *FLC* exist (below), only *FRI* has been characterized, because allelic variation at *FRI* is a major determinant of the flowering habit (i.e. annual versus winter-annual) among natural *Arabidopsis* ecotypes (Johanson *et al.*, 2000; Lee *et al.*, 1993).

The mechanistic relationships among the autonomous-pathway genes, and between these genes and *FRI*, have not been well characterized. Koornneef *et al.* (1998a) explored these relationships through genetic epistasis experiments. Mutations in *FY* did

not further enhance the late flowering conferred by loss of *FCA* function, suggesting that the two genes have a close functional relationship. In contrast, mutations in two other autonomous-pathway genes, *FPA* and *FVE*, greatly enhanced the lateness of *fca* and *fy* mutants. These genetic interactions were subsequently found to be reflected at the level of *FLC* RNA and protein expression, which were enhanced in *fpa/fca*, *fve/fca*, and *fve/fy* double mutants relative to the respective single mutants (Rouse *et al.*, 2002). These findings could indicate that *FPA/FVE* and *FCA/FY* comprise two, partially redundant, mechanisms of *FLC* repression. However, this type of analysis is contingent on mutations creating a complete loss of function, and this has not yet been demonstrated for all of these genes. Loss of *FLC* function completely suppresses the late-flowering phenotype conferred either by *FRI*, or by loss of function at least of *fve*, *ld*, *fpa*, or *fca* (Michaels and Amasino, 2001). Thus, *FRI* and these autonomous-pathway genes likely act in flowering solely through mediation of *FLC* activity. The activation of *FLC* by *FRI* is epistatic to repression of *FLC* by autonomous-pathway genes (Michaels and Amasino, 2000). This is consistent with a mechanism whereby *FRI* limits the activity of the autonomous pathway, possibly through the negative regulation of one or more components.

The flowering-promotive effect of cold, termed vernalization, is mediated largely through repression of inhibitory *FLC* activity. This also occurs at the RNA level (Michaels and Amasino, 1999; Sheldon *et al.*, 1999), and probably at the transcriptional level, as cold is not sufficient to overcome the repression of flowering associated with constitutive expression of *FLC* in transgenic plants (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). The molecular process(es) involved in vernalization-associated down-regulation of *FLC* is completely unknown. However, it is not likely to directly

involve *FRI* or the autonomous-pathway genes; the evidence for this is that a long period of cold is fully effective to abrogate the late flowering phenotype of mutants lacking activities of both *FRI* and any of the known autonomous-pathway genes (Michaels and Amasino, 2000). Although vernalization by nature should involve a temperature-sensitive mechanism, no molecular components of such a mechanism have been definitively identified. Moreover, although pathways of cold signalling in *Arabidopsis* are becoming increasingly well characterized, the involvement of known cold-signalling components in vernalization has generally not been explored. This is, at least in part, because most studies of cold signalling have been carried out in 'lab strains' of *Arabidopsis* [e.g. Columbia (Col), Landsberg *erecta* (L*er*)] that, because they lack effective *FRI* and/or *FLC* alleles, do not exhibit strong *FLC* activity and typically flower soon after germination irrespective of cold (Johanson *et al.*, 2000; Koornneef *et al.*, 1994; Lee *et al.*, 1994b). The well-known *CBF* family of transcription factors, which act as molecular 'switches' to induce many elements of the cold acclimation response (Gilmour *et al.*, 2000), do not seem to be involved in vernalization. Constitutive expression of *CBF1* or *CBF3* in a late-flowering genetic background containing active *FRI* and *FLC* alleles, although sufficient to activate cold-responsive genes, did not greatly affect flowering time or *FLC* expression (Liu *et al.*, 2002; our unpublished results).

Once *FLC* is down-regulated in vernalized plants, repression is maintained through an epigenetic mechanism involving the *VRN2* gene (Gendall *et al.*, 2001). The cold-associated down-regulation of *FLC* is not greatly affected by loss of *VRN2* function, indicating that this gene probably is not important for initial suppression of *FLC*. *VRN2* encodes a protein with sequence similarity to a member of the Polycomb-group protein

class, which has been best characterized in *Drosophila*. These proteins are components of large complexes that reinforce the transcriptionally suppressed state of homeotic genes, potentially by packaging and/or maintaining chromatin in states less accessible to transcriptional machinery (Pirrotta, 1997). Similarly, it is likely that *VRN2* functions in some way to reduce accessibility of the *FLC* gene, as *FLC* chromatin in *vrn2* mutants exhibits increased DNase sensitivity relative to that of wild-type plants, following cold treatment (Gendall *et al.*, 2001). That chromatin structure is intimately involved in flowering and vernalization, was previously shown by the strong effect on flowering conferred by disruption of processes tied to chromatin dynamics, including DNA methylation (Finnegan *et al.*, 1996; Finnegan, 1998; Ronemus *et al.*, 1996) and histone deacetylation (Tian and Chen, 2001), especially in genotypes with a winter-annual flowering habit (Burn *et al.*, 1993). Transgenic plants in which endogenous DNA methylation was disrupted exhibited decreased *FLC* expression in the absence of a vernalizing cold treatment (Sheldon *et al.*, 1999), indicating that appropriate chromatin structure is crucial for the maintenance of *FLC* expression in non-vernalized plants, as well as its suppression in vernalized plants.

As a first step to characterize the mechanism of flowering repression involving *FLC*, and how this mechanism is negatively regulated by cold, we carried out a genetic screen designed to identify positive regulators of *FLC*. Here we present the genetic and molecular analyses of one of these regulators, designated *VERNALIZATION INDEPENDENCE4*.

Material and Methods:

Plant material and growth conditions

Introgression line Col:*FRI*^{SF2} consists of the *FRI* locus from ecotype San Feliu-2 (*FRISF2*) introgressed into the Columbia (Col) ecotype through six successive backcrosses and made homozygous by self-pollination (Lee *et al.*, 1994b). Line FN231 contains a fast-neutron *flc* allele isolated in the Col:*FRI*^{SF2} background, and is identical with *flc-1* described by Michaels and Amasino (1999). Line FN235, containing a fast-neutron *fri* allele isolated in the Col:*FRI*^{SF2} background, is as described by Michaels and Amasino (1999). The *ld-1* mutant in the Col background was obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. Standard growth conditions were 22 °C under 100-180 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ of cool white fluorescent lighting and 16-h light/8-h dark (long-day) or 8-h light/16-h dark (short-day) photoperiods. For vernalizing cold treatments, seeds were surface-sterilized, placed on agar-solidified germination medium as described by van Nocker *et al.* (2000), and grown at 4 °C under SD photoperiods. To evaluate flowering time, plants were grown individually in 5.7 X 5.7 X 7.5 cm pots. Plant transformations used the floral dip method of Clough and Bent (1998) and *Agrobacterium* strain ABI.

Mutagenesis and screening

For T-DNA mutagenesis, a binary vector designated pPZP201:BAR, containing a 5' mannopine synthase/glufosinate resistance/3' octopine synthase cassette cloned into the *Sma*I site of pPZP201 (Hajdukiewicz *et al.*, 1994), was introduced into Col:*FRI*^{SF2}

plants. Seeds from infiltrated plants (T1 seeds) were subjected to a vernalizing cold treatment, transferred to soil for further growth, and herbicide-resistant T1 plants were allowed to self-pollinate and set seed. Seeds from approximately 500 T1 plants were pooled. Approximately 5000 T2 plants from each pool were screened for early flowering in the absence of cold. Fast-neutron mutagenesis and screening were described by Michaels and Amasino (1999). *FLC* RNA expression was evaluated in approximately 14-day-old progeny (T3 or M3) plants grown without a cold treatment in SD conditions. To test for genetic complementation of the *flc* or *fri* mutations, T3 or M3 individuals were crossed with lines FN231 and FN235, and flowering time was evaluated in F1 progeny.

Molecular techniques

DNA was isolated essentially as described by Murray and Thompson (1980); RNA was isolated as described by Liu *et al.* (2002). DNA and RNA gel-blot analyses were carried out as described by Liu *et al.* (2002). The probe for gel-blot analyses of *VIP4* was a 432-bp fragment amplified from genomic DNA using primers CVN4-F1 (5'..ATGGACGAAAGGAGAGTGAAAG..3') and CVN4-R1 (5'..GGAATCAGAATAT GAGACGGAAG..3'); the probe for gel-blot analysis of *FLC* was a 510-bp RT-PCR product corresponding to *FLC* coding region but excluding the conserved MADS-domain. This segment of the *FLC* gene does not exhibit significant sequence homology with any other *Arabidopsis* gene. For inverse-PCR, 200 ng of restriction endonuclease-digested genomic DNA was purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA), and subsequently incubated with 10 u T4 DNA ligase (Roche, Indianapolis, IN, USA) in a final reaction volume of 30 µl at 16 °C overnight.

DNA was amplified directly from 2 μl of the ligation mixture using Advantage cDNA Polymerase Mix (Clontech, Palo Alto, CA, USA).

For identification of *VIP4* cDNAs, shoot apex cDNA libraries were constructed. Vernalized and non-vernalized Col:*FRI*^{SF2} plants were grown under SD conditions, and when plants had formed 20-25 rosette leaves, 1-2 mm-thick sections containing the shoot apex were excised. Library construction utilized the ZAP Express XR system (Stratagene, La Jolla, CA, USA). Library A contained approximately 1.5-8.0 kbp cDNAs and had a primary titre of 6.25 X 10^6 recombinants; library B contained approximately 0.5-3.0 kbp cDNAs and had a primary titre of 4.25 X 10^6 recombinants.

Construction of transgenic Arabidopsis lines

For molecular complementation analysis, the bacteriophage P1 clone MAF19 was obtained from the Kazusa DNA Research Institute (Yana, Kisarazu, Chiba, Japan), amplified in *E. coli*, purified using the Qiagen Plasmid Midi Kit, and subjected to restriction with *Nsi*I. An approximate 7.1 kb fragment containing the *VIP4* transcriptional unit and adjacent intergenic regions was cloned into the *Pst*I site of binary vector PZP212 (Hajdukiewicz *et al.*, 1994), and introduced into the *vip4-1* mutant background. For antisense expression and constitutive expression, a DNA segment containing the *VIP4* transcribed region was amplified by PCR using primers CVN4-F1 and CVN4-R2 (5'..AGGCAAACACAAGCTCACTATC..3'), and cloned into the *Bam*HI site of binary vector pPZP201:BAR:35S in reverse (for antisense expression) or forward (for constitutive expression) orientations. The pPZP201:BAR:35S plasmid was engineered by

inserting the cauliflower mosaic virus (CaMV) *35S* promoter from plasmid pBI121 (Clontech) into the *Xba*I site of pPZP201:BAR (above).

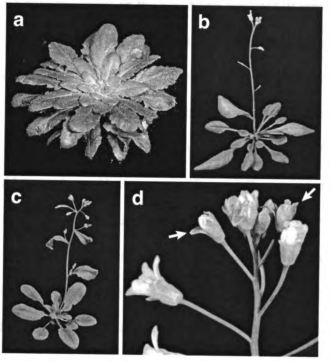
Results:

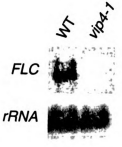
A genetic screen for activators of *FLC*

To identify potential activators of *FLC*, we mutagenized the winter-annual, Col:*FRI* ^{SF2} (hereafter referred to as 'wild-type') genetic background and screened for recessive mutations that conferred cold-independent, early flowering. Early flowering lines were re-screened by assaying for reduced *FLC* RNA expression in seedlings, where *FLC* RNA is typically easily detectable (below). To eliminate further consideration of lines with mutations in the *FLC* and *FRI* genes, mutants were also used in genetic complementation analysis with lines FN231 and FN235, carrying loss-of-function mutations in the *FLC* and *FRI* genes, respectively. Early flowering lines that exhibited reduced *FLC* RNA expression, and that were not likely to represent new alleles of *FLC* or *FRI*, were sorted into allelic groups through complementation analysis. This strategy resulted in the identification of several complementation groups representing mutants that we designated *vernalization independence* (*vip*) mutants. The *vip4* group, represented by two T-DNA alleles and one fast-neutron allele, was selected for further study (Figure 1). *FLC* expression was not detectable in plants carrying the T-DNA allele *vip4-1*, as determined by gel-blot analysis of seedling RNAs, indicating that *VIP4* is a strong activator of *FLC* (Figure 1b).

Figure 1. **(a)** Phenotype of the wild-type, Col:*FRI* ^{SF2} introgression line and a *vip4* mutant.
(i) Wild-type plant grown in the absence of cold. Plant is 8-weeks-old and has produced approximately 60 leaves.
(ii) Wild-type plant grown after a vernalizing cold treatment and flowering after approximately 3 weeks.
(iii) A *vip4-1* mutant plant grown in the absence of cold, and flowering after approximately 3 weeks.
(iv) Inflorescence from a *vip4-1* mutant plant. Morphologically abnormal flowers where sepals fail to enclose the developing floral buds are indicated (arrows).

(b) Analysis of *FLC* RNA expression in wild-type (WT) and *vip4* mutant plants. RNA was extracted from aerial portions of 14-day-old, wild-type and *vip4-1* seedlings and analysed by gel blotting using an *FLC* probe as described in Experimental procedures. The membrane was subsequently stripped and reprobed with an *18S rDNA* probe to indicate the integrity and relative quantity of total RNA in each lane.

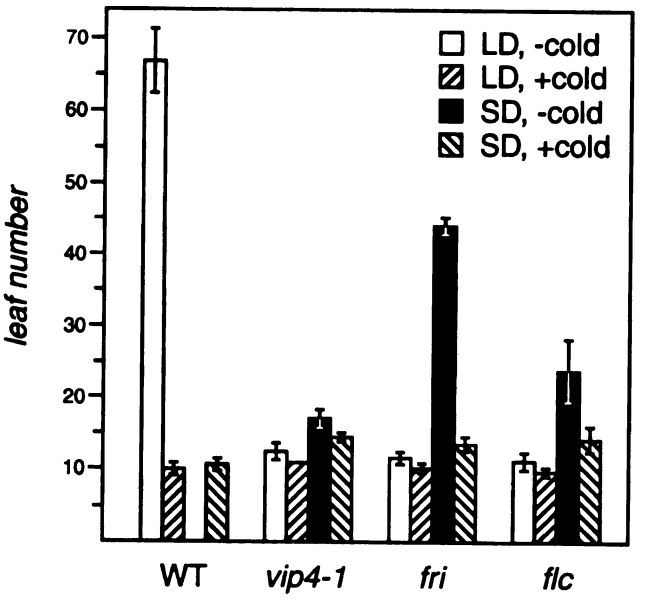

a
b
c
d


WT
vip4-1
FLC
rRNA

To address the relationship between *VIP4*, *FLC*, and *FRI*, we evaluated the effects of a vernalizing cold treatment on the flowering response of *vip4-1* relative to that of wild-type, *flc*, and *fri* plants (Figure 2). Flowering time was measured from a developmental perspective, as the total number of leaves produced on the primary stem. When grown under inductive (long-day) photoperiods in the absence of cold, *vip4* mutants flowered at approximately the same time as the *flc* and *fri* mutants, and vernalized wild-type plants. However, significant differences were apparent when plants were grown under non-inductive (short-day) photoperiods, where the promotive activity of genes acting through perception of inductive photoperiods is expected to be minimized. We found that *flc* mutants flowered earlier (23.9 ± 4.4 leaves) than *fri* mutants (44.1 ± 1.2 leaves), suggesting that *FLC* retains a small degree of activity even in the absence of *FRI* function, and this is in accordance with previous observations (Michaels and Amasino, 2001). However, under these conditions, *vip4-1* plants flowered even earlier (17.1 ± 1.3 leaves) than *flc* plants. This indicates that *VIP4* may also repress flowering outside of its positive regulation of *FLC*. Also, similar to previous observations, cold reduced the flowering time of *flc* mutants suggesting that vernalization targets *FLC*-independent as well as *FLC*-dependent mechanisms (Michaels and Amasino, 2001). However, even *vip4-1* plants showed a slight acceleration of flowering in response to cold, and vernalized wild-type plants flowered significantly earlier (10.7 ± 0.9 leaves) than did *vip4-1* plants grown in the absence of cold (Figure 2). This suggests that, if *vip4-1* is a null mutation, vernalization also involves a *vip4*-independent mechanism.

Figure 2. Flowering time of wild-type plants, *vip4* mutants, and mutants lacking activity of *FRI* or *FLC*.

Wild-type (WT) plants, *vip4-1* mutants, and the FN231 and FN235 mutants lacking activity of *FLC* or *FRI*, respectively, were grown without a cold treatment (- cold), or after a 70-day cold treatment (+ cold) under long-day (LD) or short-day (SD) photoperiods, as described in Experimental procedures. 'Leaf number' indicates the total number of rosette and cauline leaves produced. Data is the mean and standard deviation for at least 12 plants. Wild-type plants grown in SD without cold did not flower during the course of this experiment.


leaf number
70
60
50
40
30
20
10
LD, -cold
LD, +cold
SD, -cold
SD, +cold
WT
vip4-1
fri
flc

In addition to the flowering-time phenotype, *vip4* plants exhibit defects in floral morphology. Among these is a widening of medial sepals, such that sepals typically fail to enclose the remainder of the floral bud in the latest stages of floral development (Figure 1a). Petals are narrower than in wild-type flowers, and occasionally are greatly reduced in size. Stamens are often reduced in number to four or five. No defect in carpel morphology was apparent, and flowers are typically fully fertile. No additional phenotypic defects were obvious in *vip4* mutants.

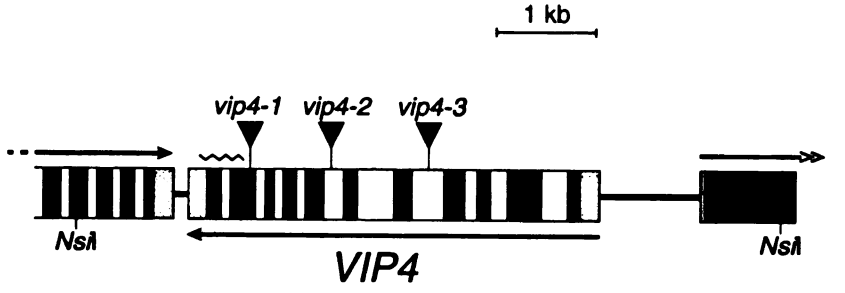
Cloning and identification of the *VIP4* gene

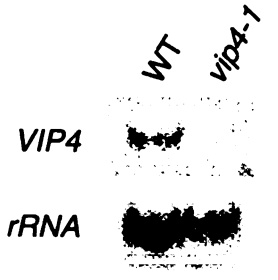
Because segregation data indicated that the *vip4-1* mutation might be due to T-DNA integration, we recovered genomic DNA flanking the T-DNA by inverse-PCR and found that the T-DNA was inserted into the transcribed region of a predicted gene near the bottom of chromosome V, designated *At5g61150* (Figure 3a). Subsequent characterization of the *At5g61150* region of *vip4-2* and *vip4-3* plants indicated the presence in the transcribed region of a T-DNA for *vip4-2*, and a large genomic insertion, originating from the top of chromosome V, for *vip4-3* (Figure 3a). In *vip4-1* plants, RNAs hybridizing with an *At5g61150* probe failed to accumulate to levels detectable by gel blotting of total RNAs, whereas these RNAs were readily detectable in wild-type plants (Figure 3b). The observed size of the transcript was approximately 2.4 kb, consistent with the size derived from the annotation of the *At5g61150* intron/exon structure provided by the Arabidopsis Genome Initiative. This predicted structure was

Figure 3. (**a**) Depiction of the region of chromosome V encompassing the *VIP4* gene.

The direction of transcription for *VIP4* and two adjacent genes is indicated by arrows. Introns are shown as white boxes, whereas exons are shown as black (translated region) or grey (untranslated region) boxes. Only the proximal portion of adjacent genes are depicted; for these, intron/exon structure is shown as annotated by the Arabidopsis Genome Initiative, and the extent of untranslated regions is based on EST sequences in the GenBank/EMBL databases. The position of the inserted DNA for the *vip4-1*, *vip4-2*, and *vip4-3* alleles is indicated. A zig-zagged line in the 3' region of the *VIP4* gene indicates the probe used for RNA gel blot analyses (below). The *Nsi*I sites delineating the region used to complement the *vip4-1* mutation are indicated.

(b) Analysis of *VIP4* RNA expression in wild-type (WT) and *vip4* mutant plants.RNA was extracted from aerial portions of 14-day-old, wild-type and *vip4-1* seedlings and analysed by gel blotting using a *VIP4* probe as described in Experimental procedures. The membrane was subsequently stripped and reprobed with an *18S rDNA* probe to indicate the integrity and relative quantity of total RNA in each lane.


1 kb
vip4-1
vip4-2
vip4-3
NsiI
NsiI
VIP4


WT
vip4-1
VIP4
rRNA

confirmed by isolation and sequencing of several cDNAs from libraries prepared from Col:*FRI* ^{SF2} shoot apices (data not shown).

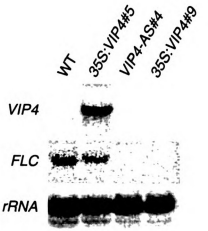
To confirm that we had identified the *VIP4* gene, we introduced the entire *At5g61150* transcriptional unit, plus immediately adjacent genomic regions, into the *vip4-1* background, through *Agrobacterium*-mediated transformation. Primary transformants (T1 plants) were grown either in the absence of cold, or after a vernalizing cold treatment. All of the 20 T1 plants recovered in both cases were phenotypically indistinguishable from wild-type plants, producing at least 60 leaves before flowering in the absence of cold, flowering very early when given a cold treatment, and exhibiting normal floral morphology (Figure 4a, and data not shown). In non-vernalized progeny of a representative T1 plant, both *VIP4* and *FLC* RNAs were expressed to levels similar to that seen in the wild-type plant (data not shown). As additional evidence that *At5g61150* is *VIP4*, we disrupted expression of the *At5g61150* gene in wild-type plants through antisense RNA expression. For this experiment, we engineered a transgene in which the part of *At5g61150* corresponding to the translated and 3' regions, including introns, was expressed in 3' to 5' orientation from the *35S* CaMV promoter. Approximately one-third of the > 100 T1 plants recovered flowered very early in the absence of cold, and produced flowers with a *vip4*-like phenotype (Figure 4a, and data not shown). Finally, in transgenic plants engineered to express the *At5g61150* transcribed region in the 5' to 3' orientation from the *35S* promoter (*35S:VIP4*; see below), early flowering, *vip4*-like plants appeared with high frequency (approximately one-third of > 100 T1 plants) (Figure 4a). The vernalization-independent early flowering of the *VIP4*-antisense and *35S:VIP4*

Figure 4. Molecular complementation of the *vip4-1* mutation and manipulation of *VIP4* expression in transgenic plants.

(a) (i, ii) Transgenic, *vip4-1* mutant plants carrying an introduced copy of the *VIP4* gene, grown without a cold treatment (i), or after a vernalizing cold treatment (ii). Plant shown in (i) is 6-weeks-old and has produced approximately 45 leaves. (iii, iv) Transgenic, wild-type plants expressing *VIP4* antisense RNA (iii) or expressing the *VIP4* gene from the *35S* promoter (iv) grown without a cold treatment.

(b) Analysis of *VIP4* and *FLC* RNA expression in wild-type and transgenic plants. *VIP4* and *FLC* RNA expression was evaluated in wild-type (WT) plants, a representative, late-flowering transgenic plant expressing the *VIP4* gene from the *35S* promoter (*35S:VIP4#5*), and representative, early flowering transgenic plants expressing *VIP4* antisense RNA (*VIP4-AS#4*) or expressing the *VIP4* gene from the *35S* promoter (*35S:VIP4#9*). RNA was extracted from rosette leaves of non-vernalized plants grown in LD photoperiods and analysed by gel blotting, using *VIP4* and *FLC* probes. Blots were subsequently stripped and reprobed with an *18S rDNA* probe to indicate the integrity and relative quantity of total RNA in each lane.


a
b
c
d


WT
35S:VIP4#5
VIP4-AS#4
35S:VIP4#9
VIP4
FLC
rRNA

plants was presumably due to suppression of the endogenous *VIP4* gene, as *VIP4* RNA did not accumulate to detectable levels in any of the several plants assayed (Figure 4b). In addition, in contrast to non-transgenic, wild-type plants, *FLC* RNA was not detectable in leaf tissues of these early flowering, *VIP4*-antisense and *35S:VIP4* plants (Figure 4b), indicating that early flowering was mediated at least partly through loss of *FLC* expression.

The *VIP4* gene encodes a 633-residue, 72-kDa protein with a predicted pI of 4.4 (data not shown). Almost one-half of the residues are charged (Glu, Asp, His, Lys, Arg) and thus the VIP4 protein is highly hydrophilic; this hydrophilicity is most apparent in extensive amino-terminal and carboxyl-terminal regions (data not shown). The VIP4 protein does not exhibit any motif currently defined in the PROSITE Dictionary of Protein Sites and Patterns. However, predominantly within its less hydrophilic central domain, VIP4 exhibits sequence homology with the Leo1 protein from *Saccharomyces cerevisiae*, and other hydrophilic proteins of unknown function from *Saccharomyces pombe*, *C. elegans*, and *Drosophila* (23-29% identity over 239-311-amino acid segments; data not shown). VIP4 does not exhibit strong homology with any other protein predicted to be encoded by the *Arabidopsis* genome, and proteins homologous to VIP4 have not been reported from other plant species.

We used RNA gel blotting to analyze the general spatial expression pattern of *VIP4* in non-vernalized plants. We found that *VIP4* was expressed throughout the plant, with the potential exception of rosette leaves (Figure 5a). We subsequently used RT-PCR to confirm that *VIP4* was expressed in these tissues as well (data not shown). This

Figure 5. Analysis of *VIP4* and *FLC* RNA expression in various organs and tissues of wild-type plants, in non-vernalized and vernalized wild-type plants, and in various genetic backgrounds.

(a) Expression in seedlings (SL), shoot apices (SA), rosette leaves (RL), cauline leaves (CL), inflorescence apices (IA), flowers (F), stems (S), and roots (R) of non-vernalized plants. In order to obtain reproductive tissues for analysis, plants were grown under long-day (LD) photoperiods.

(b) Expression in aerial portions of 14-day-old seedlings grown without a cold treatment (non-vernalized; NV), or after a 40-day cold treatment (vernalized; V) under short-day (SD) photoperiods.

(c) Expression in aerial portions of 14-day-old Columbia (Col), wild-type Col:FRI^{SF2} (WT) and *ld-1* (Col:*ld-1*) seedlings grown without a cold treatment under short-day (SD) photoperiods.RNAs were analysed by gel blotting using *VIP4* and *FLC* probes. Blots were subsequently stripped and reprobed with an *18S rDNA* probe to indicate the integrity and relative quantity of total RNA in each lane.

expression pattern generally paralleled that of *FLC*, which was also expressed ubiquitously, but at very low levels in the leaves (Figure 5a). A search of current databases of expressed sequence tags (ESTs) resulted in the identification of a single EST (BE527160) originating from developing seeds, indicating that *VIP4* is expressed in seed tissues as well.

To determine if the suppression of *FLC* RNA expression associated with vernalization might be mediated through suppression of *VIP4*, we evaluated *VIP4* RNA expression in vernalized and non-vernalized seedlings. As shown in Figure 5b, *VIP4* RNA was expressed to similar levels irrespective of the vernalization status. The effectiveness of the cold treatment given to these plants was evident by the decrease of *FLC* RNA to non-detectable levels (Figure 5b). This suggests that *VIP4* is insufficient to activate *FLC* in vernalized plants, and that modulation of *VIP4* RNA expression is unlikely to be involved in the vernalization response.

Molecular analysis of *VIP4* function

We further characterized the relationship between *VIP4*, *FRI*, and an autonomous-pathway gene, *LD*, through analysis of molecular epistasis. As previously reported (Michaels and Amasino, 1999), we did not detect *FLC* RNA expression in the Col ecotype lacking activity of *FRI*, but found that it was expressed to readily detectable levels in the Col:FRI^{SF2} line, and in an *ld* mutant in the Col background, which lacks activity of both *FRI* and *LD* (Figure 5c). In contrast, *VIP4* RNA was expressed to similar levels in all three genotypes (Figure 5c). That *VIP4* RNA expression was similar between

Col and Col:*FRI*^{SF2} indicates that *VIP4* is not likely to mediate the activation of *FLC* expression by *FRI*. Likewise, that *VIP4* expression was similar between an *ld* mutant and its wild-type genetic background Col indicates that the de-repression of *FLC* conferred by loss of *LD* function is also unlikely to be mediated through *VIP4*. We also found that *FRI* RNA expression is similar between Col:*FRI*^{SF2} and the *vip4* mutant (Figure 1, chapter 5), indicating that *VIP4* probably does not activate *FLC* through regulating *FRI* expression.

To help define the role of *VIP4* and especially its relationship to *FLC*, we evaluated the effects of enhanced expression of *VIP4* in transgenic Col:*FRI*^{SF2} plants. Several plants expressing high levels of *VIP4* RNA were identified from a *35S:VIP4* T1 population grown in the absence of cold (Figure 4b). This RNA was apparently processed to the same extent as the endogenous *VIP4* RNA, as evidenced by its co-migration with the *VIP4* transcript from wild-type plants (data not shown). These *35S:VIP4* T1 plants were phenotypically similar to wild-type plants with respect to flowering time and floral morphology (data not shown). Although *VIP4* RNA accumulated to high levels in leaf tissues of these plants, *FLC* RNA expression was not enhanced in leaves, relative to its levels in wild-type plants (Figure 4b), suggesting that ectopic *VIP4* activity was not sufficient to activate *FLC*, even in the absence of vernalization.

Discussion

As a first step towards understanding the mechanism of *FLC*-mediated flowering repression and its negative regulation by cold, we are taking a genetic approach to

identify components of floral-repressive mechanisms that act, at least partly, through promotion of *FLC* expression. Although several genetic efforts have already been carried out to identify regulators of flowering, these have mainly focused on recessive mutations that delay flowering, and thus most of the genes identified are assumed to act in a flowering-promotive capacity (Koornneef *et al.*, 1991; Lee *et al.*, 1994a; Redei, 1962). Several genes that act as floral repressors have also been identified (see below), largely through associated developmental pleiotropy, but these genes have been characterized only in the common 'lab strains' or ecotypes of *Arabidopsis* that do not normally exhibit strong *FLC* activity. Our use of a synthetic genetic background, containing an active *FRI* locus from a natural, winter-annual ecotype, introgressed into the Col genotype (Lee *et al.*, 1994b), permits rigorous genetic analysis of *FLC*-associated repressive mechanism(s), while simultaneously permitting full utilization of currently available Arabidopsis genomics tools.

The genetic and molecular analysis of *VIP4* demonstrates that it acts as a repressor of flowering at least partly through its ability to strongly activate *FLC*. Our current knowledge of flowering is consistent with *FLC* being regulated predominantly through at least two mechanisms or pathways (Michaels and Amasino, 1999). One mechanism involves the autonomous-pathway genes, which repress *FLC* expression, and *FRI*, which acts antagonistically to the autonomous pathway (Simpson *et al.*, 1999), possibly by limiting the activity of one or more components. At least a second mechanism must be proposed to promote *FLC* expression, based on the observation that, in plants lacking activity of the autonomous pathway, *FLC* is strongly expressed even in the absence of *FRI*. Because *FLC* expression is repressed by cold even in the absence of

FRI and/or autonomous pathway function, vernalization likely acts to limit the activity of this second mechanism.

VIP4 could be hypothesized to occupy any of a number of positions with respect to these pathways. *VIP4* RNA levels were not affected by loss of function of *FRI* or *LD*, indicating that, if *VIP4* mediates activation of *FLC* by *FRI* and/or de-repression of *FLC* by loss of *LD* activity, such a mechanism would have to involve changes in VIP4 protein activity, or changes in RNA levels within restricted tissues. Our observation that *vip4* mutants flower much earlier than *fri* null mutants also suggests that *VIP4* does not act in flowering exclusively with *FRI* as a co-activator of *FLC*. Thus, it is possible that *VIP4* acts independently of these genes in a distinct mechanism required for *FLC* expression in the absence of cold. We found that increasing *VIP4* RNA expression was not sufficient to further activate *FLC*, even in non-vernalized plants where other elements necessary for *FLC* expression are active. Also, *vip4* mutations appear to be completely recessive (data not shown). The lack of gene dosage effect is consistent with *VIP4* acting as one non-limiting component of a more extensive mechanism. Obvious candidates for other potential components are represented by the several allelic groups of *vip* mutations that we have identified through our genetic approach.

A flowering-repressive mechanism involving *VIP4* could function in several possible capacities. For example, because the 'vernalized state' is not maintained through meiosis (i.e. the requirement for cold is re-set in each generation; Lang, 1965), this mechanism could act to re-establish *FLC* expression in the developing embryo, possibly by disrupting the epigenetic repressive mechanism involving *VRN2*. If so, then this might

be reflected by decreased accessibility of *FLC* chromatin in the *vip4* mutant, relative to that in wild-type plants. Another possibility is that *VIP4* acts in a hypothetical pathway of vernalization cold signalling, maintaining it in an 'off' state. However, if this is the case, then *VIP4* is unlikely to act as a general suppressor of cold-signalling pathways, a role hypothesized for the *HOS1* gene (Lee *et al.*, 2001), because unlike *hos1* mutants, *vip4* plants exhibited neither ectopic expression of a representative cold-responsive gene, *COR78*, nor enhanced freezing tolerance as measured by electrolyte leakage assays (our unpublished results).

Irrespective of its nature, the flowering-repressive mechanism involving *VIP4* could be deactivated by cold through the negative regulation of one or more components. The observation that *VIP4* RNA is expressed to equivalent levels in both non-vernalized and vernalized plants suggests that if *VIP4* itself were a cold-regulated component, regulation would either be mediated at the level of VIP4 protein activity, or at the RNA level within a restricted subset of tissues. However, in this respect, it is noteworthy that the subtle floral defects seen in plants lacking *VIP4* activity are not observed in vernalized, wild-type plants, suggesting that *VIP4* maintains activity in vernalized plants, at least in floral tissues.

The *VIP4* protein exhibits sequence homology with yeast Leo1 and proteins from *Drosophila* and *C. elegans*; in addition, the highly hydrophilic nature of these proteins is conserved. These observations suggest that these proteins could function in analogous molecular mechanisms. Of these proteins, only Leo1 has been characterized. High-throughput, proteomic analyses suggest that Leo1 physically interacts with multiple

protein partners in several cellular contexts (Gavin *et al.*, 2002; Ito *et al.*, 2001). This protein has been shown to exhibit an ATP-sensitive interaction with the 19S 'cap' of the proteasome (Verma *et al.*, 2000), and is a component of the Paf1 transcriptional complex, which is required for full expression of a subset of yeast genes (Mueller and Jaehning, 2002). It is noteworthy that the defects in floral morphology seen in *vip4* mutants are not observed in mutants or natural ecotypes lacking *FLC* activity (Michaels and Amasino, 1999), suggesting that the role of *VIP4* in floral development is mediated outside of its relationship with *FLC*. Thus, *VIP4* likely acts as a common component of distinct developmental mechanisms, possibly through interactions with multiple protein partners.

The observation that *vip4* mutants flower earlier than *flc* null mutants indicates that *VIP4* regulates flowering-time genes in addition to *FLC*. These hypothetical target(s) could have a role in GA biosynthesis or sensitivity, or in the perception of photoperiod, as current models of flowering predict that such mechanisms would influence flowering outside of pathway(s) involving *FLC* (Simpson *et al.*, 1999). An especially attractive candidate is *FLM* [also known as *AGL27* (Alvarez-Buylla *et al.*, 2000) or *MAF1* (Ratcliffe *et al.*, 2001)], which encodes a MADS-box protein highly related to *FLC*, and acts as a floral repressor through a mechanism that is likely independent of *FLC* (Ratcliffe *et al.*, 2001; Scortecci *et al.*, 2001). Other possibilities include *AGL31*, a tandemly repeated cluster of four genes which also encode proteins highly related to *FLC* (Alvarez-Buylla *et al.*, 2000; Scortecci *et al.*, 2001).

A more speculative candidate for an additional target of *VIP4* regulation is the MADS-box gene *AGAMOUS* (*AG*), which has functions in floral organ and meristem

identity (Mizukami and Ma, 1997). Ectopic expression of *AG* is associated with early flowering, and in some cases, floral defects similar to that observed for *vip4* (Gómez-Mena *et al.*, 2001; Mizukami and Ma, 1997; Serrano-Cartagena *et al.*, 2000; our unpublished results). If this were the case, then *VIP4* would be included in an expanding class of gene acting both in floral repression and negative regulation of *AG*. These genes include *CURLY LEAF* (*CLF*), *WAVY LEAVES and COTYLEDONS* (*WLC*), *INCURVATA 2* (*ICU2*), *EMBRYONIC FLOWER* (*EMF*)*1* and *2*, and *EARLY BOLTING IN SHORT DAYS* (*EBS*) (Chen *et al.*, 1997; Gómez-Mena *et al.*, 2001; Goodrich *et al.*, 1997; Serrano-Cartagena *et al.*, 2000; Simpson *et al.*, 1999). Interestingly, Goodrich *et al.* (1997) found that the ectopic activity of *AGAMOUS* could not explain the full degree of early flowering of the *clf-2* mutant plants, and suggested that *CLF* also regulates other flowering gene(s). It is tempting to speculate that *CLF*, and perhaps these other genes as well, might play a role as regulators of *FLC*. As these genes have been characterized only in laboratory strains of Arabidopsis that lack *FRI* function, and therefore express *FLC* only weakly, their potential to regulate *FLC* remains unclear. The possibility that these genes are involved in vernalization is intriguing because at least *CLF* and another member of this class, *EMF2*, encode Polycomb-group-like proteins (Goodrich *et al.*, 1997; Yoshida *et al.*, 2001), suggesting that they participate in epigenetic regulation of gene activity. To test this possibility will require the introduction of the respective mutations into genetic backgrounds that normally express *FLC*. Alternatively, new alleles of these genes may be identified through further characterization of other *vip* allelic groups.

References

Alvarez-Buylla, E.R., Liljegren, S.J., Pelaz, S., Gold, S.J., Burgeff, C., Ditta, G.S., Vergara-Silva, F., and Yanofsky, M.F. (2000) MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cell, roots and trichomes. Plant J. 24: 457-466.

Aukerman, M.J. and Amasino, R.M. (1996) Molecular genetic analysis of flowering time in Arabidopsis. In: Seminars in Developmental Biology, Vol. 7 (Amasino, R.M., ed.). Cambridge: Academic Press, pp. 427-434.

Burn, J.E., Bagnall, D.J., Metzger, J.M., Dennis, E.S., and Peacock, W.J. (1993) DNA methylation, vernalization, and the initiation of flowering. Proc. Natl. Acad. Sci. USA 90: 287-291.

Chen, L., Cheng, J.C., Castle, L., and Sung, Z.R. (1997) *EMF* genes regulate Arabidopsis inflorescence development. Plant Cell 9: 2011-2024.

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

Finnegan, E.J. (1998) DNA methylation and the promotion of flowering by vernalization. Proc. Natl. Acad. Sci. USA 95: 5824-5829.

Finnegan, E.J., Peacock, W.J., and Dennis, E.S. (1996) Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development. Proc. Natl. Acad. Sci. USA 93: 8449-8454.

Gavin, A.C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M.A., Copley, R.R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G.. (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415: 141-147.

Gendall, A.R., Levy, Y.Y., Wilson, A., and Dean, C. (2001) The *VERNALIZATION2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell 107: 525-535.

Gilmour, S.J., Sebolt, A.M., Salzar, M.P., Everard, J.D., and Thomashow, M.F. (2000) Overexpression of the Arabidopsis *CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 124: 1845-1865.

Gomez-Mena, C., Pineiro, M., Franco-Zorrilla, J.M., Salinas, J., Coupland, G., and Martinez-Zapater, J.M. (2001) *early bolting in short days*: An Arabidopsis mutation that causes early flowering and partially suppresses the floral phenotype of leafy. Plant Cell 13: 1011-1024.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997) A polycomb-group gene regulates homeotic gene expression in Arabidopsis. Nature 386: 44-51.

Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol. Biol. 25: 989-994.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl. Acad. Sci. USA 98, 4569-4574.

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

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

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

Koornneef, M., Blankestijn-de Vries, H., Hanhart, C., Soppe, W., and Peeters, T. (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. Plant J. 6: 911-919.

Koornneef, M., Hanhart, C.J., and van der Veen, J.H. (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol. General Genet. 229: 57-66.

Lang, A. (1965) The physiology of flower initiation. In: Encyclopedia of Plant Physiology (Ruhland, W., ed.). Berlin: Springer-Verlag, pp. 1371-1536.

Lee, I., Aukerman, M.J., Gore, S.L., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C., Feldmann, K.A., and Amasino, R.M. (1994a) Isolation of *LUMINIDEPENDENS* a gene involved in the control of flowering time in Arabidopsis. Plant Cell 6: 75-83.

Lee, I., Bleecker, A., and Amasino, R.M. (1993) Analysis of naturally occurring late flowering in *Arabidopsis thaliana*. Mol. General Genet. 237: 171-176.

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

Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B., and Zhu, J.-K. (2001) The Arabidopsis *HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. Genes Dev. 15: 912-924.

Liu, J., Gilmour, S.J., Thomashow, M.F., and van Nocker, S. (2002) Cold signalling associated with vernalization in *Arabidopsis thaliana* does not involve *CBF1* or abscisic acid. Physiol. Plant. 114, 125-134.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997) *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding motifs. Cell 89: 737-745.

Michaels, S.D. and Amasino, R.M. (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 Amasino, R.M. (2000) Memories of winter: vernalization and the competence to flower. Plant Cell Environ. 23: 1145-1153.

Michaels, S.D. and Amasino, R.M. (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.

Mizukami, Y. and Ma, H. (1997) Determination of Arabidopsis floral meristem identity by *AGAMOUS*. Plant Cell 9: 393-408.

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

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular-weight DNA. Nucl. Acids Res. 8: 4321-4325.

Pirrotta, V. (1997) PcG complexes and chromatin silencing. Curr. Opin. Genet. Dev. 7: 249-258.

Ratcliffe, O.J., Nadzan, G.C., Reuber, T.L., and Riechmann, J.L. (2001) Regulation of flowering in Arabidopsis by an *FLC* homologue. Plant Physiol. 126: 122-132.

Redei, G.P. (1962) Supervital mutants of Arabidopsis. Genetics 47: 443-460.

Ronemus, M., Galbiati, M.K., Ticknor, C., Chen, J., and Dellaporta, S. (1996) Demethylation-induced developmental pleiotropy in Arabidopsis. Science 273: 654-657.

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

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

Scortecci, K.C., Michaels, S.D., and Amasino, R.M. (2001) Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering. Plant J. 26: 229-236.

Serrano-Cartagena, J.S., Candela, H., Robles, P., Ponce, M.R., PeÂ rez-PeÂ rez, J.M., Piqueras, P., and Micol, J.L. (2000) Genetic analysis of *incurvata* mutants reveals three independent genetic operations at work in Arabidopsis leaf morphogenesis. Genetics 156: 1363-1377.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (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., Gendall, A.R., and Dean, C. (1999) When to switch to flowering. Annu. Rev. Cell Dev. Biol. 99: 519-550.

Tian, L. and Chen, Z.J. (2001) Blocking histone deacetylation in Arabidopsis induces pleiotropic effects on plant gene regulation and development. Proc. Natl. Acad. Sci. USA 98: 200-205.

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

Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R.J. (2000) Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol. Biol. Cell. 11: 3425-3439.

Yoshida, N., Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R., and Takahashi, S. (2001) *EMBRYONIC FLOWER2*, a novel polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis. Plant Cell 13: 2471-2481.

Chapter 3

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.*

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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.

Introduction

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 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, Wellensiek, 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'-TTGCAGGTGGAAGTAGTGCCTC-3') and F27B13.7 R2 (5'-TGTCATCAGAGACAC TAGCAAGTCG-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'-GCTCAATCAGTCATTGCAC TC-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 F1 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. M2 seed was collected in pools each representing ~1000 M1 individuals. Approximately 5000 plants from each of 24 fast neutron-derived M2 families and 20 EMS-derived M2 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 M2 plants to wild type. For genetic complementation analysis, mutants were grown at 18 °C, under which conditions all mutants were fertile.

Positional cloning of the *VIP3* gene utilized F2 progeny of a single F1 individual derived from a cross between *vip3* and introgression line L*er*:FRI^{SF2}:FLC^{SF2}. 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 (http://www.arabidopsis.org/cereon/index.html; courtesy of S. Rounsley) and noted in Fig 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'-AAAGGATCCATGAAACTCGCAGG TCTGAAATCG-3') and F27B13.7RBam2 (5'-AAAGGATCCGAATTGTTCATGAGTA ATCATAGAGC-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'-AAAGGATCCAATGCCATCCCTGACATGG CTTGC-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°C in a boiling water bath, and clarifying by centrifugation for 5 min at 12,000 x *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 (Fig 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 (Fig 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 (Fig 1A, c). In addition, flowers of *vip3* plants exhibited

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.


A
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b
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d
e
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g
h
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p
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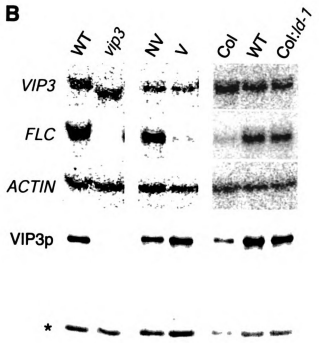
p
s


B
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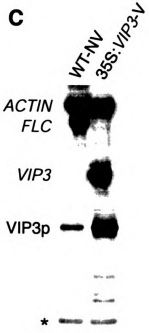
C
WT-NV
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FLC
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abnormalities of organs in the outer three whorls (Fig 1A, h). Sepals typically had irregular, translucent margins, and lateral sepals were always reduced in size. Petals were also reduced 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 °C), 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 (Fig 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 flowering response of the *vip3* mutant relative to that of wild-type plants and plants carrying a null mutation in *FLC* (Fig 2A 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 (Fig 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 (Fig 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

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.

leaf number
A
B
C
SGC
C
SD+C
SD
SGC
C
SD+C
SGC
SD
C
SD+C
WT
vip3
flc-3
vip3
vip3/fri
vip3/fri/fld

leaves) than wild-type plants given a 70-day cold treatment (10.7 ± 0.9 leaves; Fig 2). A 70-day cold treatment reduced flowering time of *vip3* plants to that of vernalized wild-type plants (Fig 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; Fig 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; Fig 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 (Fig 2A 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 (Fig 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; Fig 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.

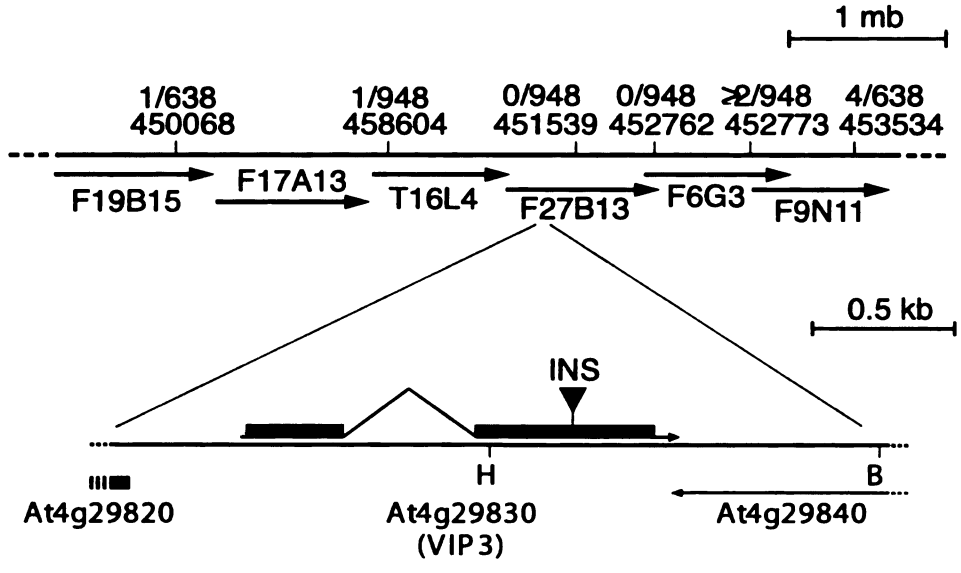
To determine if *FRI* has any flowering-repressive effect in a *vip3* genetic background, we evaluated the effect of the strong loss-of-function fri^{Col} allele on flowering time of *vip3* plants. When grown under long-day photoperiods, there was no significant difference in flowering time between *vip3* single mutants and *vip3/fri* double mutants (Fig 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; Fig 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 (Fig 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; Fig 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 (Fig 1B). DNA and RNA gel-blot analyses of the adjacent genes, At4g29820 and

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 ß-strands, here labeled A, B, and C, is based on the structure determined for the Gß protein. A fourth ß-strand found in WD motifs, strand D, is not strongly conserved at the amino acid sequence level and is not indicated.

A



B

```
                A                    B          C
SIEN AHEDSV WAATWV PATRDRPA    LLLTGS LDE TVKLWR PDELDLVRTNT  57
     GHSLGV AALAAH PSGI        IAASSS IDS FVRVFD VDTNATIAVLE  99
     APPSEV WGMQFE PKGTI       LAVAGG SSA SVKLWD TASWRLISTLS  155
     SSKKFV LSVAWS PNGK        RLACGS MDG TICVFD VDRSKLLHQLE  197
     GHNMPV RSLVFS PVDPR       VLFSGS DDG HVNMHD ARGKTLLGSMS  240
     NHNDQV WSVAFR PPGGTGVRAG RLASVS DDK SVSLYD YS  321
     GHxxxV xxVxFx PDG~        xLASGS xDx TIKVWD ~
     A    I   L W  SNS          IVTAG     SVRLFN
              I L  DSP          VL SA      L IY
              C I               FI C       A
                V
```

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 (Fig 1B). This protein was not detectable in equivalent extracts from *vip3* plants (Fig 1B). Also, there were no immunoreactive protein species of larger or smaller molecular mass unique to *vip3* extracts (Fig 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 (Fig 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 (Fig 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; Fig 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 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 (Fig 1B). In addition, to determine if the promotive activity of *VIP3* on *FLC* expression is mediated via activation of *FRI*, we evaluated *FRI* RNA expression in wild-type plants and in the

vip3 mutant. We did not observe a significant change in *FRI* RNA expression between wild-type and *vip3* (Figure 1, chapter 5). 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 (Fig 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 (Fig 1A, e). In contrast, nonvernalized wild-type plants grown under similar conditions produced at least 60 rosette leaves without flowering (Fig 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 (Fig 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 (Fig 1C). Even in these *VIP3*-expressing plants, *FLC* expression was not detectable (Fig 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:

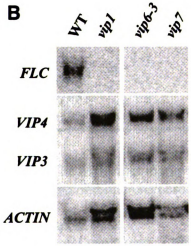
The phenotype of the *vip3* mutant was similar to that of plants with mutations in the previously identified flowering-time gene *VIP4* (Zhang and van Nocker, 2002). For example, *vip4* mutants do not express detectable levels of *FLC*, flower earlier than an *FLC* null mutant, and exhibit defects in 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

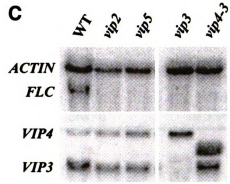
class of gene with similar roles in flowering timing and floral development could exist in Arabidopsis. To attempt to define the extent of this potential gene class, we mapped all of the seven *vip3/4*-like mutations recovered in our screens to a limited region of the genome, and those plants harboring mutations on the same chromosome were subjected to genetic complementation analysis (Table 1 and Table 2). In addition to *VIP3* and *VIP4*, five new loci were identified, which we tentatively designated *VIP1*, *VIP2*, *VIP5*, *VIP6*, and *VIP7* (Table 1 and Table 2; Fig 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 co-segregated with early flowering in the small mapping populations and with a frequency expected for Mendelian segregation of a single, recessive locus (Fig 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 (Fig 4E). The exception was *vip2*, originating in an EMS-mutagenized population, which flowered slightly later (Fig 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 (Fig 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).

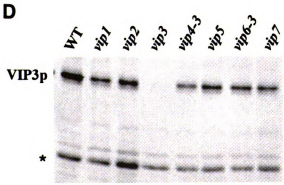
To help clarify the potential relationships among these genes, we analyzed *VIP3*

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.


A
a
b
c
d
e
f
g
h
i
j


B
WT
vip1
vip6-3
vip7
FLC
VIP4
VIP3
ACTIN


C
WT
vip2
vip5
vip3
vip4-3
ACTIN
FLC
VIP4
VIP3


D
WT
vip1
vip2
vip3
vip4-3
vip5
vip6-3
vip7
VIP3p
*

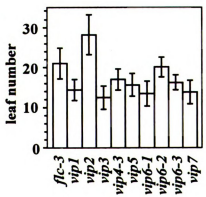

E
leaf number
30
20
10
0
flc-3
vip1
vip2
vip3
vip4-3
vip5
vip6-1
vip6-2
vip6-3
vip7

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).

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 F1 progeny from the crosses indicated. In all cases where crosses produced mutant F1 progeny, only mutant individuals were observed in the corresponding F1 progeny. Selection for crosses to be analyzed was on the basis of map position determined for the respective mutations (see text).

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 (Fig 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 (Fig 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 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 Gß shows that each of the seven WD motif units takes the form of four antiparallel ß-strands, with the seven repeated WD motifs forming a symmetrical structure termed a ß-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 Gß 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 ß-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 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* mutants. 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.

References

Aukerman, M. J., and Amasino, R.M. (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., Wilson, A., and Dean, C. (1996) Arabidopsis mutants showing an altered response to vernalization. Plant J. 10: 637-644.

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

Gendall, A.R., Levy, Y.Y, Wilson, A., and Dean, C. (2001) The *VERNALIZATION2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell 107: 525-535.

Harlow, E., and Lane, D. (1988) Antibodies -- a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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

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

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

Koornneef, M., Alonso-Blanco, C., Peeters, A.J.M., and Soppe, W. (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., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M., and Lee, I. (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev. 14: 2366-2376.

Lee, I. and Amasino, R. M. (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., Michaels D.D., Masshardt A.S., and Amasino, R.M. (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., Mesnage, S., Mylne, J.S., Gendall, A.R., and Dean, C. (2002) Multiple roles of Arabidopsis *VRN1* in vernalization and flowering time control. Science 297: 243-246.

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

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997) *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding motifs. Cell 89: 737-745.

Michaels, S.D. and Amasino, R.M. (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 Amasino, R.M. (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 Jaehning, J.A. (2002) Ctr9, Rtf1, and Leo1 are components of the Paf1/RNA polymerase II complex. Mol. Cell. Biol. 22: 1971-1980.

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

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

Samach, A, Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000) Distinct roles of *CONSTANS* target genes in reproductive development of Arabidopsis. Science 288: 1613-1616.

Schomburg, F.M., Patton, D.A., Meinke, D.W., and Amasino, R.M. (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., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (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 Dean, C. (2002) Arabidopsis, the rosetta stone of flowering time? Science 296: 285-289.

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

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

van Nocker, S., Muszynski, M., Briggs, K., and Amasino, R. M. (2000) Characterization of a gene from *Zea mays* related to the Arabidopsis flowering-time gene *LUMINIDEPENDENS.* Plant Mol. Biol. 44: 07-122.

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 van Nocker, S. (2002) The *VERNALIZATION INDEPENDENCE4* gene encodes a novel regulator of *FLOWERING LOCUS C.* Plant J. 31: 663-667.

Chapter 4

A plant transcriptional regulatory mechanism related to the yeast Paf1/RNA polymerase II complex contains VERNALIZATION INDEPENDENCE proteins

Flowering time data of *vip3 vip4*, *vip4 vip5* and *vip4 vip6* double mutants, anti-VIP4 antibody characterization, and immunocoprecipitation of VIP3, VIP4 and VIP6 were contributed to the paper by Oh, S., **Zhang, H.**, Ludwig, P., and van Nocker, S. (2004) A mechanism related to the yeast transcriptional regulator Paf1C is required for expression of the Arabidopsis *FLC/MAF* MADS-box gene family. Plant Cell 16: 2940-2953.

Abstract

The Arabidopsis *VERNALIZATION INDEPENDENCE* genes are essential for expression of *FLOWERING LOCUS C* (*FLC*), a major target of vernalization. Four of these *VIP*s (*VIP2*, *VIP4*, *VIP5*, *VIP6*) encode proteins similar to components of the yeast Paf1/RNA polymerase II complex (Paf1C). Considering the indistinguishable phenotype of *vip* mutants, VIPs were proposed to participate in a protein complex related to Paf1C, which has recently been shown to be involved in nucleosomal histone modifications. Genetic and biochemical approaches were used to test this hypothesis. It was found that double mutants of *vip4* and other *vip* mutants did not give an enhanced phenotype when compared with either of the single mutant parents; and VIP3, VIP4, VIP6 proteins immunocoprecipitated. Both results suggest that VIPs may be involved in an evolutionally conserved transcription regulatory mechanism to maintain gene expression in higher eukaryotic organisms.

Introduction

Flowering to set seeds is a crucial step for plants to maintain their ecological niche. Thus, this process is under delicate controls of multiple mechanisms to ensure that reproductive growth coincides with favorable conditions. For certain species, flowering does not occur or is delayed unless the plant is exposed to cold temperatures for several weeks - a phenomenon known as vernalization.

In Arabidopsis, vernalization is mediated predominantly via epigenetic silencing of the MADS-box flowering repressor gene *FLOWERING LOCUS C* (*FLC*). At least three factors, VERNALIZATION INSENSITIVE3 (VIN3), VERNALIZATION1 (VRN1) and VRN2, are necessary for initiating or maintaining the cold-associated *FLC* suppression. *VIN3* encodes a protein containing a plant homeodomain (PHD) that is often found in proteins associated with chromatin remodeling complexes (Sung and Amasino, 2004a); *VRN1* encodes a putative DNA binding protein (Levy et al., 2002); and VRN2 encodes a protein similar to the Drosophila Polycomb-group protein Su(z)12 (Gendall et al., 2001). Molecular studies suggest that these proteins may sequentially modify histone H3 within *FLC* chromatin to establish a transcriptionally silent state (Sung and Amasino, 2004a; Bastow et al., 2004). From a recent hypothetical model, histone H3 of *FLC* chromatin in the promoter and first intron regions is de-acetylated during vernalizing cold, dependent on VIN3 and unknown histone deacetylase (HDAC) activities. This hypoacetylated chromatin region may subsequently be targeted by a histone methyltransferase (HMT) mechanism mediated by VRN1 and VRN2 proteins to methylate H3 at lysine-9 (K9) and K27 residues (Sung and Amasino, 2004b). At least in

yeast and animal systems, methylation at H3K9 in euchromatic regions is associated with heterochromatin and subsequent long-term transcriptional silencing (Schultz et al., 2002).

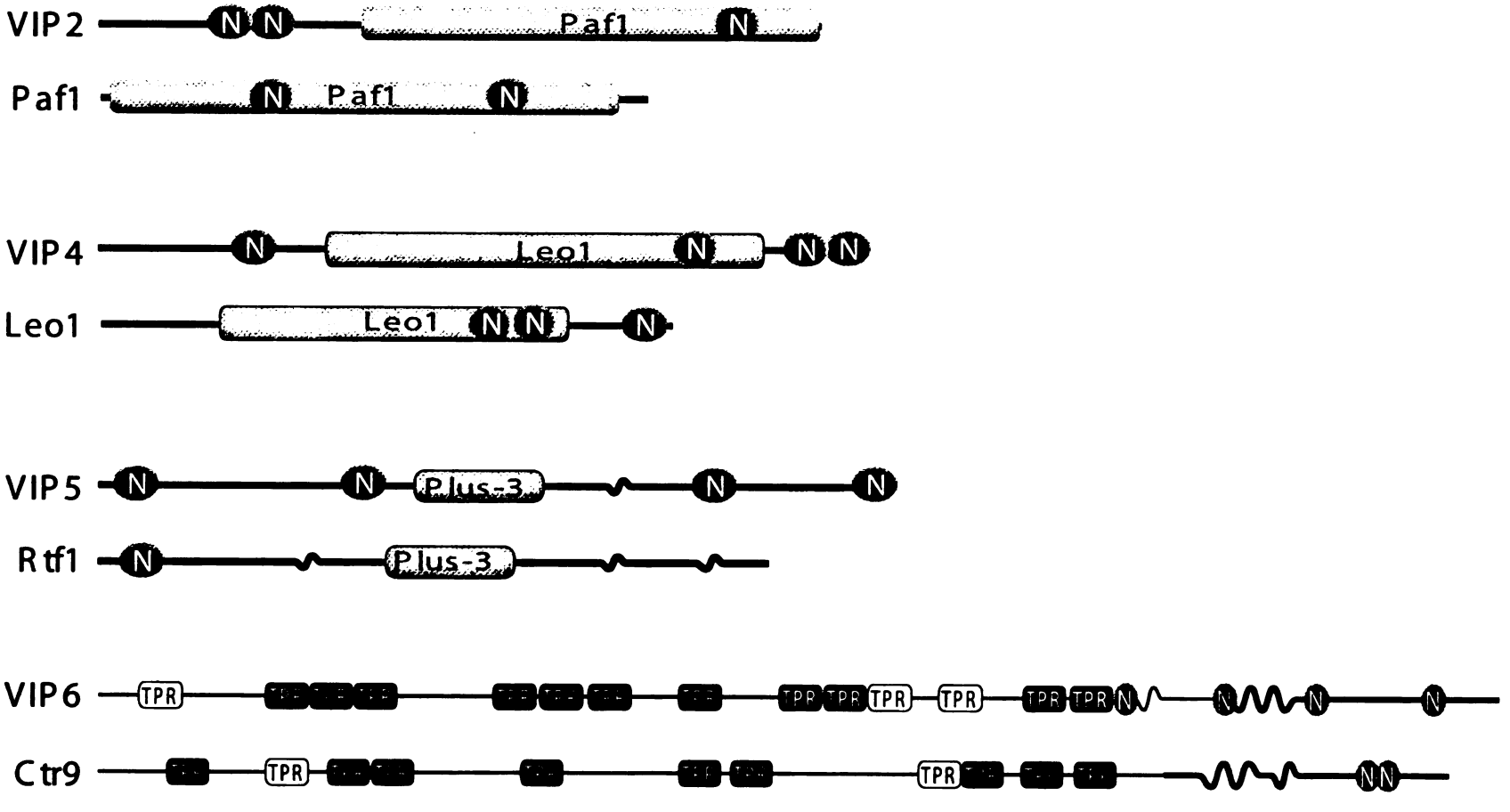
In addition to being negatively regulated by vernalization, genetic and molecular studies indicated that *FLC* is also under controls of several other mechanisms, "funneling" regulatory signals to suppress the expression of the flowering pathway integrator genes *SOC1* and *FT*, which, in turn, suppresses the floral transition (Simpson and Dean, 2002; Lee et al., 2000; Samach et al., 2000; Michaels and Amasino, 2001). An autonomous flowering pathway, including the *LUMINIDEPENDENS* (*LD*), *FCA*, *FY*, *FVE*, *FPA*, *FLOWERING LOCUS D* (*FLD*), and *FLOWERING LATE WITH KH MOTIFS* (*FLK*) genes, apparently limits the accumulation of *FLC* transcripts through different mechanisms, based on the proteins encoded by these genes. For example, *LD* encodes a protein with a homeobox domain (Lee et al., 1994a), which is often found in proteins associated with chromatin remodeling complexes (Fair et al., 2001), suggesting a role of LD in chromatin dynamics. However, in some other cases, homeodomain proteins were also shown to bind RNA (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996), indicating that LD might also be involved in posttranscriptional control. *FCA*, *FPA* and *FLK* encode proteins containing a RNA binding domain, suggesting their roles in posttranscripiton regulation as well (Macknight et al., 1997; Schomburg et al., 2001; Lim et al., 2004). *FLC* chromatin is hyperacetylated in the *fld* and *fve* mutant, suggesting that FLD and FVE may regulate flowering by histone deacetylation (He et al., 2003; Ausin et al., 2004). In contrast to the autonomous pathway genes, *FRIGIDA* (*FRI*) and *FRIGIDA*-related (*FRL*) genes positively regulate *FLC* expression (Michaels and

Amasino, 1999; Sheldon et al., 1999; Michaels et al., 2004). It is not known how FRI activates the expression of *FLC*.

A group of seven *vernalization independence* (*vip*) mutants (*vip1* - *vip7*) were recently isolated to define novel *FLC* positive regulators (Zhang and van Nocker, 2002; Zhang *et al.*, 2003). These *vip* mutants, identified from a vernalization responsive genetic background, flower early and suppress *FLC* RNA expression in the absence of vernalizing cold. The apparently identical phenotype of the *vip* mutants suggests that they may operate in the same pathway, possibly as components of the same protein complex. Besides early flowering, the *vip* mutants also show mild developmental pleiotropy, suggesting that *VIP* genes may play multiple roles in plant development (Zhang *et al.*, 2003). Molecular and genetic epistatic analysis suggested that the VIP mechanism might be independent of FRI and the autonomous pathway (Zhang et al., 2003).

Five of these *VIP* genes have been cloned. *VIP3* encodes a protein consisting almost entirely of WD motifs, which have been proposed to play a role in protein-protein interaction. *VIP2*, *VIP4*, *VIP5* and *VIP6* encode proteins with sequence homology to yeast proteins Paf1, Leo1, Rtf1 and Ctr9, respectively (Figure 1) (Ek-Ramos and van Nocker, unpublished; Zhang and van Nocker, 2002; Zhang et al., 2003; Oh et al., 2004). Together with another protein Cdc73, these yeast proteins are components of the Paf1/RNA polymerase II complex (Paf1C, here and after), which mediates full expression of a small subset of yeast genes and was proposed to be involved in transcription elongation (Shi *et al.*, 1997; Mueller and Jaehning, 2002; Krogan *et al.*, 2002). The sequence homology between these VIPs and the Paf1C components suggests

Figure 1. The homology of VIP2/Paf1, VIP4/Leo1, VIP5/Rtf1 and VIP6/Ctr9. The pictures are drawn to scale. Putative nuclear localization signals, predicted using the server at http://psort.ims.u-tokyo.ac.jp, are shown as 'N'. The homologous regions of VIP2/Paf1 and VIP4/Leo1 are shown as shaded boxes. The Plus-3 domain of VIP5/Rtf1 and the TPR repeats in VIP6/Ctr9 are indicated.
The pictures showing the homology of VIP5/Rtf1 and VIP6/Ctr9 are from Oh et al., 2004.


VIP2
N
N
Paf1
N
Paf1
N
Paf1
N
VIP4
N
Leo1
N
N
N
Leo1
Leo1
N
N
N
VIP5
N
N
Plus-3
N
N
Rtf1
N
Plus-3
VIP6
TPR
TPR
TPR
TPR
TPR
TPR
N
N
N
N
Ctr9
TPR
TPR
N
N

that VIPs might constitute a plant counterpart of Paf1C and regulate a sub-set of plant genes (including *FLC*) involved in multiple developmental processes. The presented research aimed to further characterize the VIP mechanism by determining the genetic interactions among VIPs and exploring the physical interaction among these proteins.

Material and Methods

Plant material and manipulations

The *flc* null mutant *flc-3* was a gift from Richard Amasino (University of Wisconsin). The *vip1-1*, *vip3-1*, *vip4-2*, *vip5-1*, *vip6-1* and *vip7-1* mutants are as described previously (Zhang and van Nocker, 2002; Zhang et al., 2003). The *vip2-046605* (SALK_046605) mutant was obtained from Arabidopsis Biological Resource Center at The Ohio State University (Columbus, OH) and introduced into the Col:*FRI* background (Lee et al., 1994b).

To generate transgenic plants expressing FLAG-epitope-tagged VIP3 protein, a VIP3 transcriptional unit, containing 1.2 kb of 5'/promoter DNA, was amplified from wild-type plant DNA using primers PstI-VIP3F (5'..AAACTGCAGTAACGCTCGAGCT TCTTCACCC..3') and BamHI-VIP3R (5'..AAAGGATCCTGAGTAATCATAGAGCGA TACA..3'), and cloned into the plant expression vector pHuaFLAG (unpublished). The pHuaFLAG vector is based on pPZP201:BAR (Zhang and van Nocker, 2002), and allows for a carboxyl-terminal translational fusion of one hexahistidine and two tandem copies of the FLAG epitope. Plants were transformed using the floral dip method (Clough and Bent, 1998) and Agrobacterium strain GV3101.

Standard growth conditions were 22 °C under 100-180 μmol/m2/sec of cool white fluorescent lighting and 16-h light/8-h dark (long-day) or 8-h light/16-h dark (short-day) photoperiods. Vernalizing cold treatments were as previously described (Zhang and van Nocker, 2002).

Double mutants construction

All double mutants were obtained using the same two-step identification scheme. The *vip4-2* mutant, containing a T-DNA conferring resistance to the herbicide glufosinate, was crossed with the other six *vip* mutants. Herbicide selection was conducted in the F2 population and herbicide resistant *vip*-like individuals were chosen for further screening. The *vip4/VIP4* individuals were identified by PCR using primers x2-allele2F (5'..CTCGA TTCAACAATGGCAGTC AAG..3') and x2-allele2R (5'.. ATTGATCCAAAGCCTTTT GATGCC..3'), which amplifies a fragment from the wild-type *VIP4* allele, but not from the *vip4-2* mutant allele. *vip4/VIP4* plants that exhibited a *vip*-like phenotype were assumed to be homozygous for the other *vip* mutation. The identified *vip/vip vip4/VIP4* plant was allowed to self and the *vip vip4* double mutants were then identified from the F3 progeny using the primer set mentioned above.

Immunoblot analysis

To generate VIP4 antiserum, a hexahistidine-tagged VIP4 amino-terminal portion (amino acids 1-202) was expressed in *E. coli* and affinity purified using Ni^{2+}-affinity chromatography. The anti-VIP3 and anti-VIP6 antisera were as described (Zhang et al., 2003; Oh et al., 2004). Anti-FLAG M2 monoclonal antibody was purchased from Sigma

(St. Louis, Mo.; catalog no. F-3165). For immunoblot analysis, total protein was extracted from inflorescence tissues using a protocol described previously (Zhang et al., 2003). For immunoprecipitation experiments, anti-VIP4 and anti-VIP6 IgGs were purified by elution from Protein A-agarose (Roche) using a procedure described by the manufacturer. We used protein extracts from inflorescence apices, because VIP4 and VIP6 are strongly expressed in these tissues. Approximately 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 (PMSF)] 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 carried out at 4 °C.

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

Results

Genetic epistatic analysis between *vip4* and other *vip* mutants

Epistatic analysis is a powerful tool to dissect the genetic interactions between two genes, and it has been successfully used to determine the genetic interactions among late flowering Arabidopsis mutations (Koornneef *et al.*, 1998). In epistatic analysis, a double mutant containing two mutations is constructed, and the phenotype of this double mutant is examined. The epistatic mutation refers to the mutation that completely masks the phenotype of the other one. If the mutations under investigation confer opposite signaling states of the same pathway, the epistatic mutation will be genetically downstream of the other gene; whereas if the two genes confer the same signaling state of the same pathway, the epistatic one is genetically upstream. If the two mutations confer similar phenotype and if a more severe phenotype (additive or synergistic) is observed in the double mutant versus either of the single mutants, it may imply that these two genes act in two different epistatic groups, whereas in the case that no enhanced phenotype is observed, these two genes may act in the same epistatic group. Because leaky alleles may give ambiguous results, epistatic analysis is mostly easily interpreted when both of the mutations are null alleles.

Currently, the *vip* mutant group is represented by seven genetic loci (*vip1* through *vip7*). To determine if the known *VIP*s are involved in the same mechanism, double mutants of *vip4* and other six *vips* were generated and their flowering time was measured. Under short-day conditions, all single and double mutants flowered significantly earlier than the *flc* null (Figure 2). However, there was no significant difference in flowering between the double mutants and their single mutant parents (Figure 2), and the double mutants were morphologically indistinguishable from the single mutant parents (data not

Figure 2. Combination of *vip4* with *vip1*, *vip2*, *vip3*, *vip5*, *vip6* and *vip7* mutations does not enhance the early flowering phenotype of the single mutants. The data are shown to scale. Flowering time of the double mutants is shown as gray bars. The flowering time, quantified under short-day conditions, was measured as total leaf numbers on the primary stem. Data was obtained by evaluating at least 10 plants from all genotypes except for the *vip2-046605 vip4-2*, for which only four plants were evaluated.

Total leaf number
10
20
30
40
Rc
41.8 ± 1.2
vip4-2
18.6 ± 2.1
vip1-1
18.7 ± 1.3
vip1-1 vip4-2
18.4 ± 2.0
vip2-04660S
17.2 ± 1.4
vip2-04660S vip4-2
16.8 ± 1.4
vip3-1
18.2 ± 1.4
vip3-1 vip4-2
17.4 ± 1.5
vip5-1
19.1 ± 1.8
vip5-1 vip4-2
16.7 ± 2.2
vip6-1
18.9 ± 1.8
vip6-1 vip4-2
17.9 ± 1.5
vip7-1
19.6 ± 1.7
vip7-1 vip4-2
19.8 ± 2.2

shown). This lack of synergistic effect is consistent with the hypothesis that VIPs are involved in the same mechanism.

We previously found that a mutation in *VIP2* (*vip2-1*) was associated with incomplete suppression of *FLC* (Zhang et al., 2003). The *vip2-1* allele was recovered from a population mutagenized by ethyl methanesulfonate (EMS), which typically generates point mutations and weak alleles. When grown under the same short-day conditions, the *vip2-046605* mutant flowered significantly earlier than *vip2-1* (data not shown). The *vip2-046605* mutant, with a T-DNA inserted in the 8th intron of *VIP2* (data not shown), was generated by T-DNA insertion mutagenesis (Alonso et al. 2003), which typically results in null or strong alleles. Together with the observation that *vip2-046605* flowered at about the same time as other *vip* single mutants (Figure 2), these results suggest that *vip2-046605* probably represents a strong or null allele.

Dysfunction of VIPs does not affect VIP4 protein accumulation

In Arabidopsis, VIP6 protein levels are lower in *vip1* – *vip5* mutants when compared with the wild-type, suggesting that VIPs are important for maintaining VIP6 protein levels (Oh et al., 2004). Similarly, in yeast, disruption of some Paf1C components affects the accumulation of other integral subunits of Paf1C. For example, loss of Paf1p caused decrease of Leo1, Rtf1p, Cdc73p and Ctr9p protein abundance (Squazzo et al., 2002; Mueller et al., 2004). To determine if any of the VIPs are involved in maintaining VIP4 abundance, an anti-VIP4 antibody was used to detect VIP4 protein in all other six *vip* mutants. Except for the *vip4* mutant, this VIP4 antibody specifically recognized a ~125 kDa protein species from wild-type and other *vip* mutants, indicating

that these *VIP* genes are not involved in maintaining VIP4 abundance (Figure 3). The observed molecular mass of the VIP4 protein (~125 kDa) is much larger than the size predicted from the amino acid sequence (~72 kDa), which could be due to an abnormal migration of VIP4, or posttranslational modifications on the VIP4 protein.

VIP3, VIP4 and VIP6 physically interact *in vivo*

The phenotypic similarity among the *vip* mutants, the results of the epistatic analysis, and the sequence homology between yeast Paf1C components and some VIPs all suggest that at least some VIPs, if not all, may operate in a protein complex.

As an initial effort to study the constitution of this hypothetical complex, an immunocoprecipitation experiment was carried out. Anti-VIP4 IgG immunoprecipitated a protein species from wild-type plant protein extract that was strongly immunoreactive with anti-VIP6 antisera and that migrated at the expected position for VIP6 (~ 130 kDa) (Figure 4A). No such protein was detected in immunoprecipitates from either *vip4-2* or *vip6-1* mutant protein extracts, or from immunoprecipitations with no antibody (mock) or with the pre-immune sera from wild-type plant protein extract (Figure 4A). A reciprocal immunocoprecipitation using anti-VIP6 IgG immunoprecipitated a ~125 kDa protein species that was recognized by anti-VIP4 antisera from wild-type protein extract (Figure 4B). No such protein was present in immunoprecipitates from *vip4-2* or *vip6-1* mutant protein samples, or from a mock immunoprecipitation (Figure 4B). Consistent with the observation that VIPs are essential for maintaining VIP6 abundance (Oh et al., 2004), a barely detectable amount of VIP6 was precipitated from the *vip4-2* mutant extract using anti-VIP6 IgGs.

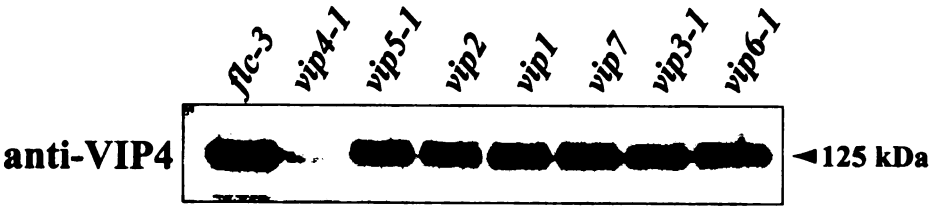


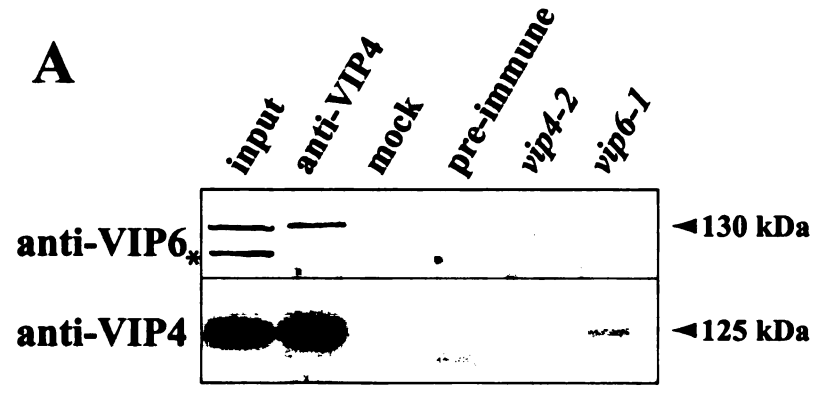
Figure 3. VIP4 protein abundance in all seven *vip* mutants. The proteins were extracted from inflorescence tissues as described in Materials and Methods. The anti-VIP4 antibody recognizes the N-terminal portion (amino acids 1 to 202) of the VIP4 protein.

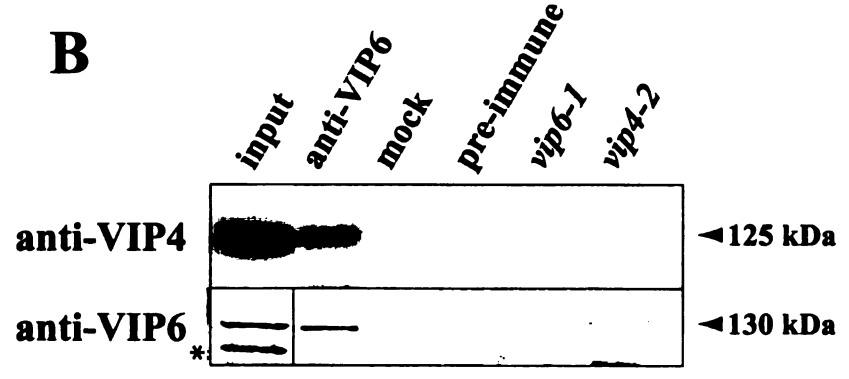
Figure 4. VIP3, VIP4 and VIP6 physically interact in vivo.

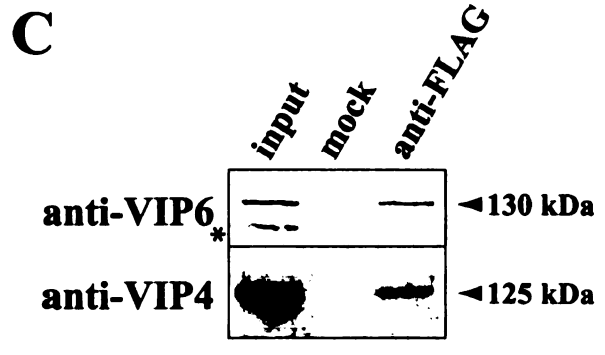
(A) and **(B)** Physical interaction between VIP4 and VIP6. Total protein from wild-type inflorescence apices (four lanes at left in each panel) was subjected to immunoprecipitation using anti-VIP4 IgGs **(A)** or anti-VIP6 IgGs **(B)**. Immunoprecipitates were analyzed by protein gel blotting using anti-VIP6 or anti-VIP4 serum as indicated at left. No immunoreactive protein was detected when immunoprecipitations were performed in the absence of IgGs (mock) or using the respective preimmune sera. Parallel immunoprecipitations were performed using extracts from the strong *vip4-2* and *vip6-1* mutants (two lanes at right in each panel).

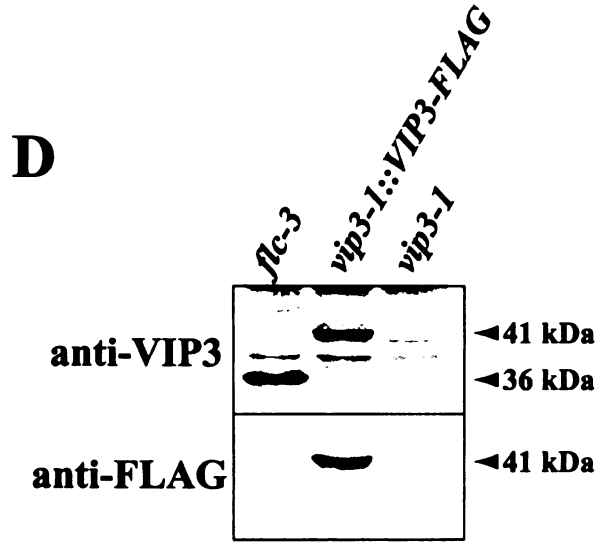
(C) Physical interaction between VIP3, and VIP4 and VIP6. Total inflorescence apex protein from *vip3-1* plants expressing a transgenic copy of FLAG-epitope-tagged VIP3 was subjected to immunoprecipitation using anti-FLAG antibody. Immunoprecipitates were analyzed by protein gel blotting using anti-VIP6 or anti-VIP4 serum as indicated at left. No immunoreactive protein was detected when immunoprecipitations were performed in the absence of antibody (mock). In each panel, an unrelated, VIP6-immunoreactive protein species present in total protein extracts is indicated (*). Immunoblots were developed using colorimetric detection (anti-VIP6) or enhanced chemiluminescence and autoradiography (anti-VIP4).

(D) Extracts from *flc* mutant plants (*flc-3*), *vip3-1* plants containing a transgenic copy of the *VIP3-FLAG* construction, and *vip3-1* plants were subjected to immunoblot analysis utilizing anti-VIP3 sera (upper panel) or FLAG antibody (lower panel).


A
input
anti-VIP4
mock
pre-immune
vip4-2
vip6-1
anti-VIP6*
anti-VIP4
130 kDa
125 kDa


B
input
anti-VIP6
mock
pre-immune
vip6-1
vip4-2
anti-VIP4
anti-VIP6*
125 kDa
130 kDa


C
input
mock
anti-FLAG
anti-VIP6*
anti-VIP4
130 kDa
125 kDa


D
fic-3
vip3-1::VIP3-FLAG
vip3-1
anti-VIP3
anti-FLAG
41 kDa
36 kDa
41 kDa

Although VIP3 does not show homology with any of the Paf1C components, the apparently indistinguishable phenotype between *vip3* and other *vip* mutants suggests that VIP3 could work in concert with VIP4 and VIP6. To test the possible physical interactions of VIP3, VIP4 and VIP6, we carried out immunocoprecipitation experiments using protein extract from a transgenic plant expressing a FLAG-epitope tagged VIP3 protein in the *vip3-1* mutant background (Figure 4D). This epitope-tagged VIP3 fusion protein fully complemented the *vip3* mutant phenotype (data not shown), suggesting its functional equivalency to the native VIP3. The anti-FLAG monoclonal antibody immunoprecipitated proteins that were strongly immunoreactive to the anti-VIP4 and anti-VIP6 antisera, with the molecular masses expected for VIP4 and VIP6 proteins. No such protein species was observed from a mock immunoprecipitation (Figure 4C).

Based on these immunocoprecipitation results, we concluded that VIP3 interacts with VIP4 and VIP6 in vivo, and that VIP3, VIP4 and VIP6 are components of a protein complex.

Discussion

For flowering, winter-annual plants must effectively override flowering suppressive mechanism(s) associated with vegetative growth via a process known as vernalization. In Arabidopsis, one of such flowering suppressive mechanisms includes at least seven *VIP* genes, which are required to maintain the expression of the MADS-box flowering suppressors *FLC* and *MAF*s (Zhang et al., 2003; Oh et al., 2004). Consistent with the hypothesis that the *FLC*-dependent vernalization mechanism might be conserved within the Brassicaceae family (Tadege *et al.*, 2001), proteins immunoreactive to anti-

VIP3, anti-VIP4 and anti-VIP6 antibodies are also found in vernalization responsive *Brassica oleracea* cultivars (Figure 5 and data not shown). Interestingly, at least VIP4 and VIP6 have clear homologues in the rice genome, whereas no FLC homologue is present, suggesting that VIPs and their targets also participate in gene expression in plant species outside of the Brassicaceae family.

Our epistatic analysis indicates that the seven known *VIP*s may regulate *FLC* through the same mechanism. No enhanced phenotype, in terms of either flowering time or plant morphology, was observed when combining mutations in *VIP4* and each of the other six *VIP* genes, suggesting that these *VIPs* comprise a single epistatic group. However, the lack of synergistic effect in the *vip4 vip6* double mutant could also be due to the dependence of VIP6 upon the presence of VIP4 (Oh et al., 2004). It is not known if there are any sub-epistatic groups exist among the *VIP*s (i.e., if some VIPs have distinct function). In yeast, although Ctr9 and Paf1 are present in the same complex, compromising either one confers a more severe phenotype than compromising Cdc73 or Rtf1 (Betz et al., 2002), suggesting that they have distinct functions. Indeed, a very recent study indicates that Paf1 and Rtf1 may be involved in mRNA polyadenylation, a function distinct from chromatin modification (see below) (Mueller et al., 2004). An analysis of the complete array of the double mutants, with all possible combinations of *vip* mutations, is needed to establish the detailed epistatic relationships, if any, within the group. However, because VIP6 accumulation is dependent on the other VIPs (i.e., VIP6 protein level is very low in other *vip* mutants) (Oh et al., 2004), it is not likely to observe an enhanced phenotype in double mutants containing the *vip6* mutation.

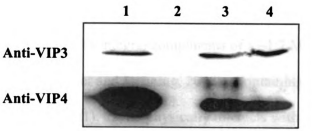


Figure 5. Protein species immunoreactive to anti-VIP3 and anti-VIP4 antibodies are present in broccoli and cauliflower. Total protein was extracted from shoot meristematic tissues as described in Materials and Methods. 1, Wild-type Arabidopsis; 2, Arabidopsis *vip3-1* (top panel) or *vip4-2* (bottom panel) mutant; 3, broccoli; 4, cauliflower.

The genetic epistatic analysis indicated that the VIPs might operate in a common mechanism. Given the observation that VIP4, VIP5 and VIP6 are homologous to components of yeast Paf1C, most likely the VIPs are involved in a plant protein complex. The immunocoprecipitation experiment showed that at least VIP3, VIP4, and VIP6 physically interact *in vivo* (Figure 4), indicating that these VIPs may indeed operate in a protein complex, consistent with our previous hypothesis (Zhang and van Nocker, 2002; Zhang et al., 2003). Whereas no VIP3 homologue is present in yeast, VIP4 and VIP6 yeast homologues were found as integral components of a ~1.7 MDa transcriptional complex named Paf1C (Mueller and Jaehning, 2002). Containing three additional yeast proteins (Rtf1, Cdc73, and Paf1), Paf1C physically interacts with the initiating and elongating forms of RNA polymerase II (Mueller and Jaehning, 2002). If VIPs indeed constitute a plant counterpart of Paf1C, we would expect that VIP2 and VIP5, homologues of Paf1 and Rtf1, respectively, also show physical interactions with VIP4 and VIP6. However, we cannot exclude the possibility that some components may have been removed from the complex during evolution. The physical interaction of VIP3 with VIP4 and VIP6 suggests that the structure of Paf1C might be elaborated in plants. On one hand, it is possible that new components (e.g., VIP3) may have been recruited to the Paf1C during the course of plant evolution to provide more specific controls for expanded genome coding capacity and increased chromatin structure complexity. On the other hand, some specific components might have been disassociated from the complex and evolved into distinct mechanisms. Preliminary studies of an Arabidopsis mutant carrying a mutation in the yeast Cdc73 homologous gene did not show a *vip* mutant phenotype nor suppress *FRI*, but had a strong effect on the photoperiodic response (van

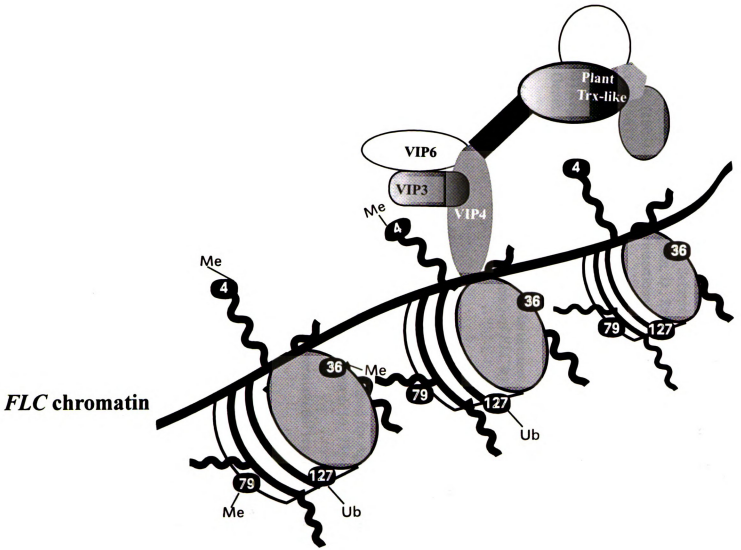
Nocker, unpublished). It is possible that the AtCDC73 specifically targets the *MAF*s of the *FLC/MAF* gene family, but not *FLC*.

How VIPs regulate *FLC* is highly speculative. Recent studies indicate that, rather than being simply present as scaffold proteins, at least some of the Paf1C components are required for different histone modifiers to set up a 'histone code' on their target genes (Gerber and Shilatifard, 2003). The 'histone code' is a combination of different covalent modifications (*e.g.*, phosphorylation, methylation, acetylation, ubiquitination, sumoylation) on nucleosomal histones to epigenetically determine the transcriptional state of a gene (for review of the 'histone code' hypothesis, see Jenuwein and Allis, 2001). The Paf1C subunits Paf1p and Rtf1p may regulate the activity of Rad6-Bre1 complex to ubiquinate histone H2B on lysine-123 (K123) in the promoter regions (Ng et al., 2003; Wood et al., 2003). H2BK123 ubiquitination is a prerequisite step for the subsequent methylation of histone H3K4 and K79 by the histone methyltransferases (HMT) Set1 and Dot1, respectively. In an ubiquinated H2BK123-independent manner, H3K36 can be methylated by another HMT, Set2 (Ng et al., 2003). At least methylation at H3K4 and H3K79 are usually associated with transcriptionally active genes (Hampsey and Reinberg, 2003). The recruitment of all three HMTs mentioned above to chromatin requires the presence of Paf1C, and mutations in *Rtf1* or *Ctr9* cause a global methylation defect in yeast (Krogan et al., 2003a, b). Intriguingly, the human trx-related MLL (mixed lineage leukemia) protein, which carries out H3K4 methylation at Hox loci in vivo, is related to the yeast HMT Set1 (Milne et al., 2002).

Given the homology between VIPs and Paf1C components, the physical interaction of VIP4 and VIP6, and the existence of homologs of additional Paf1C

components in the Arabidopsis genome, it is possible that VIPs represent a plant counterpart of the Paf1C. Probably, VIPs may function in concert with a plant Trx-like mechanism to label genes (possibly including *FLC* or an *FLC* activator) as transcriptionally active, by assisting histone methylation on H3K4/K36/K79 (Figure 6). However, immunoblotting analysis of chromatin histone-enriched proteins from *vip3*, *vip4*, *vip5* and *vip6* mutants using antisera specific for histone H3 methylated at K4, K36, or K79 did not detect any significant difference in apparent abundance of modified histones when compared with the wild-type control extracts (Oh et al., 2004), suggesting that, unlike their yeast homologs, VIPs apparently do not affect histone methylation in a genome-wise (global) manner, at least on the H3K4/K36/K79 residues.

Figure 6. A model for the VIP mechanism. VIPs may represent a plant version of Paf1C. This hypothetical VIP complex may function in concert with a plant Trithorax-like mechanism to activate *FLC* during vegetative stage, by assisting histone posttranslational modifications


Plant
Trx-like
VIP6
VIP3
VIP4
Me
4
36
79
127
Ub
FLC chromatin

References

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genomewide insertional mutagenesis of *Arabidopsis thaliana*. Science 301: 653-657.

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004) Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat Genet. 36: 162-6.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., and Dean, C. (2004) Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature 427: 164-167.

Betz, J.L., Chang, M., Washburn, T.M., Porter, S.E., Mueller, C.L., and Jaehning JA. (2002) Phenotypic analysis of Paf1/RNA polymerase II complex mutations reveals connections to cell cycle regulation, protein synthesis, and lipid and nucleic acid metabolism. Mol. Genet. Genomics 268: 272-285.

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

Dubnau, J., and Struhl, G. (1996) RNA recognition and translational regulation by a homeodomain protein. Nature 379: 694-699.

Fair, K., Anderson, M., Bulanova, E., Mi, H., Tropschug, M., and Diaz, M.O. (2001) Protein interactions of the MLL PHD fingers modulate MLL target gene regulation in human cells. Mol. Cell. Biol. 21: 3589–3597.

Gendall, A.R., Levy, Y.Y, Wilson, A., and Dean, C. (2001) The *VERNALIZATION2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell 107: 525-535.

Gerber, M. and Shilatifard, A. (2003) Transcriptional elongation by RNA polymerase II and histone methylation. J. Biol. Chem. 278: 26303-26306.

Hampsey M., and Reinberg D. (2003) Tails of intrigue: Phosphorylation of RNA polymerase II mediates histone methylation. Cell 113: 429-432.

Harlow, E., and Lane, D. (1988) Antibodies -- a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

He, Y., Michaels, S., and Amasino, R. (2003) Regulation of flowering time by histone acetylation in Arabidopsis. Science 302: 1751-1754.

Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. Science 293: 1074-1080.

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

Krogan, N.J., Kim, M., Ahn, S.H., Zhong, G., Kobor, M.S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J.F. (2002) RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: a targeted proteomics approach. Mol. Cell. Biol. 22: 6979-6992.

Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M., Greenblatt, J.F., and Shilatifard, A. (2003a) The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol. Cell. 11: 721-729.

Krogan, N.J., Kim, M., Tong, A., Golshani, A., Cagney, G., Canadien, V., Richards, D.P., Beattie, B.K., Emili, A., Boone, C., Shilatifard, A., Buratowski, S., and Greenblatt, J. (2003b) Methylation of histone H3 by Set2 in Saccharomyces cerevisiae is linked to transcriptional elongation by RNA polymerase II. Mol. Cell Biol. 23: 4207-4218.

Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M., and Lee, I. (2000) The *AGAMOUS-LIKE 20* MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev. 14: 2366-2376.

Lee, I., Aukerman, M.J., Gore, S.L., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C., Feldmann, K.A., and Amasino, R.M. (1994a) Isolation of *LUMINIDEPENDENS*: A gene involved in the control of flowering time in Arabidopsis. Plant Cell 6: 75-83.

Lee, I., Michaels D.D., Masshardt A.S., and Amasino, R.M. (1994b) 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., Mesnage, S., Mylne, J.S., Gendall, A.R., and Dean, C. (2002) Multiple roles of Arabidopsis *VRN1* in vernalization and flowering time control. Science 297: 243-246.

Lim, M.H., Kim, J., Kim, Y.S., Chung, K.S., Seo, Y.H., Lee, I., Kim, J., Hong, C.B., Kim, H.J., and Park, C.M. (2004) A new Arabidopsis gene, *FLK*, encodes an RNA binding protein with K homology motifs and regulates flowering time via *FLOWERING LOCUS C*. Plant Cell 16: 731-40.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997) *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding motifs. Cell 89: 737-745.

Michaels, S.D. and Amasino, R.M. (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 Amasino, R.M. (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.

Michaels, S.D., Bezerra, I.C., and Amasino, R.M. (2004) *FRIGIDA*-related genes are required for the winter-annual habit in Arabidopsis. Proc. Natl. Acad. Sci. USA 101: 3281-3285.

Milne, T.A., Briggs, S.D., Brock, H.W., Martin, M.E., Gibbs, D., Allis, C.D., and Hess, J.L. (2002) MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol. Cell 10: 1107-1117.

Mueller, C.L. and Jaehning, J.A. (2002) Ctr9, Rtf1, and Leo1 Are Components of the Paf1/RNA Polymerase II Complex. Mol. Cell. Biol. 22: 1971-1980.

Mueller, C.L., Porter, S.E., Hoffman, M.G., and Jaehning, J.A. (2004) The Paf1 complex has functions independent of actively transcribing RNA polymerase II. Mol. Cell. 14: 447-56.

Ng, H.H., Dole, S., and Struhl, K. (2003) The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. J. Biol. Chem. 278: 33625-33628.

Oh, S., Zhang, H., Ludwig, P., and van Nocker, S. (2004) A mechanism related to the yeast transcriptional regulator Paf1C is required for expression of the Arabidopsis *FLC/MAF* MADS-box gene family. Plant Cell 16: 2940-2953.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W.J., and Jackle, H. (1996) RNA binding and translational suppression by bicoid, Nature 379:746-749.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000) Distinct roles of *CONSTANS* target genes in reproductive development of Arabidopsis. Science 288:1613-1616.

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

Schultz, D.C., Ayyanathan, K., Negorev, D., Maul, G.G., and Rauscher, F.J. 3rd. (2002) SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev. 16: 919-932.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999) The *FLF* MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell 11: 445-458.

Shi, X., Chang, M., Wolf, A.J., Chang, C.-H., Frazer-Abel, A.A. Wade, P.A., Burton, Z.F., and Jaehning, J.A. (1997) Cdc73a and Paf1p are found in a novel RNA polymerase II- containing complex distinct from the Srbp-containing holoenzyme. Mol. Cell. Biol. 17: 1160-1169.

Simpson, G.G. and Dean, C. (2002) Arabidopsis, the Rosetta stone of flowering time? Science 296: 285-289.

Squazzo, S.L., Costa, P.J., Lindstrom, D.L., Kumer, K.E., Simic. R., Jennings, J.L., Link, A.J., Arndt, K.M., and Hartzog, G.A. (2002) The Paf1 complex physically and functionally associates with transcription elongation factors *in vivo*. EMBO J. 21: 1764-1774.

Sung, S., and Amasino, R.M. (2004a). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature 427: 159-164.

Sung, S., and Amasino, R.M. (2004b) Vernalization and epigenetics: how plants remember winter. Curr Opin Plant Biol. 7: 4-10.

Tadege, M., Sheldon, C.C., Helliwell, C.A., Stoutjesdijk, P., Dennis, E.S., and Peacock, W.J. (2001) Control of flowering time by *FLC* orthologues in *Brassica napus*. Plant J. 28: 545-553

Wood, A., Krogan, N.J., Dover, J., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Golshani, A., Zhang, Y., Greenblatt, J.F., Johnston, M., and Shilatifard, A. (2003). Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. Mol. Cell 11: 267–274.

Zhang, H. and van Nocker, S. (2002) The *VERNALIZATION INDEPENDENCE4* gene encodes a novel regulator of *FLOWERING LOCUS C*. Plant J. 31: 663-673.

Zhang, H., Ransom, C., Ludwig, P., and van Nocker, S. (2003) Genetic analysis of early-flowering mutants in Arabidopsis defines a class of pleiotropic developmental regulator required for activity of the flowering-time switch *FLC*. Genetics 164: 347-358.

Chapter 5

Perspectives and future directions

Introduction

One of the most important adaptive features for biennial and winter-annual plants is vernalization, which ensures plants to flower after winter, under physiologically favorable conditions. Using Arabidopsis as a model, it was found that, after vernalizing cold, the MADS-box flowering repressor *FLOWERING LOCUS C* (*FLC*) is epigenetically silenced (Michaels and Amasino, 1999; Sheldon et al., 1999), and mutants that lost the capability of initiating or maintaining *FLC* silencing give a vernalization insensitive phenotype (Sung and Amasino, 2004; Gendall et al., 2001; Levy et al., 2002), both suggesting an essential role of *FLC* in vernalization.

My research has been focused on how *FLC* is maintained in an active state during the vegetative growth stage. Taking a forward genetic approach, I screened mutagenized winter-annual populations for Arabidopsis mutants that do not require vernalizing cold to flower. A group of seven *vernalization independence* (*vip1* - *vip7*) genes was identified as novel *FLC* activators. Genetic and molecular epistatic analysis suggested that these VIPs might represent a *FLC* regulatory mechanism distinct from that of the previously known *FLC* regulators, e.g., FRIGIDA and LUMINIDEPENDENS (Zhang and van Nocker, 2002; Zhang et al., 2003; Figure 1).

In this chapter, I will discuss the genetic approach that I adopted to identify the *VIP*s and speculation of the mechanism for these *FLC* activators. Finally, I will present preliminary results from my ongoing projects.

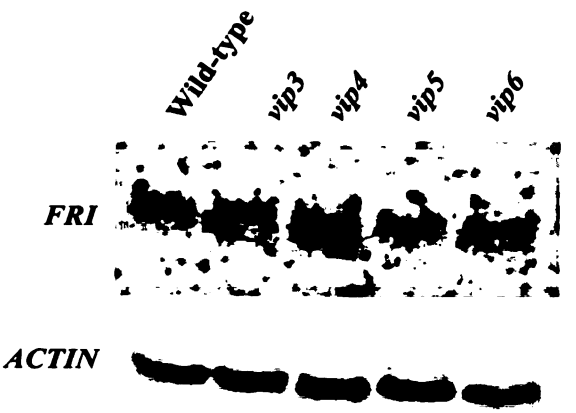


Figure 1. VIPs operate in a mechanism distinct from that of *FRIGIDA*. Total RNA was extracted from aerial portions of 14-day-old, wild-type and *vip3-1*, *vip4-2*, *vip5-1*, *vip6-1* seedlings and analyzed by gel blotting using a *FRIGIDA* probe, amplified using primers FRIP-F 5'-AAAGGATCCCATGTCCAATTATCCACCGAC-3' and FRIP-R 5'-AAAACCTGGATCCGTGACCGCTTCAAAGCA-3'. The membrane was subsequently stripped and reprobed with an *ACTIN* probe (described in Zhang et al., 2003) to indicate the integrity and relative quantity of total RNA in each lane.

A successful genetic approach to identify *FLC* regulators

The completely sequenced genome and the availability of vernalization-responsive strains make Arabidopsis an excellent model to study the molecular basis of vernalization. Different mutant screen criteria were used to target genes that are involved in different regulation mechanisms of the hypothetical "vernalization pathway". In general, screening for mutants that do not need vernalizing cold treatment to flower will most likely target genes that are able to keep the vernalization pathway in an "off" (for loss-of-function mutants) or "on" (for gain-of-function mutants) state; while screening for mutants that are insensitive to vernalizing cold will probably recover genes that are required to "turn on" (for loss-of-function mutants) or "turn off" (for gain-of-function mutants) the vernalization pathway.

The *vip*s were identified using the first mutant screening criteria, from screening populations mutagenized by fast-neutron and T-DNA insertion, which typically generate strong loss-of-function mutations. My results showed that VIPs are essential for *FLC* expression. From this point of view, VIPs define factors to keep the vernalization pathway in an "off" state. However, vernalization does not seem to operate through downregulating *VIP*s, which indicates that VIPs only act closely with vernalization, rather than being integral components of this process. It is noteworthy that *vip*s only represent a subgroup of the mutants that have been identified from my screening (Table 1). Two additional subgroups of mutants may define additional *FLC* activators, one subgroup is represented by mutants A2, C1, F1, G2, T36-1, and T46-4, which are indistinguishable from vernalized wild-type plants. The other subgroup is represented by mutants A3 and N7, which are serrated and flower almost as early as vernalized wild-

Table 1. Early flowering mutants that were identified from my screening. This table listed all 13 mutants that have been recovered from screening of a fast-neutron mutagenized population and a T-DNA insertion mutagenized population. The names in parenthesis represent specific alleles.

Fast neutron mutants	T-DNA mutants
A2	*vip4-1*
A3 (N7)	T34-1 (G2)
C1	T46-4
vip5-1	*vip6-2*
F1	
G2 (T34-1)	
N7 (A3)	
vip3-1	
vip6-1	

type plants. The existence of these 'non-*vip*' mutants suggests that *FLC* might be under the control of a regulatory network more complicate than what we thought before. Besides the *vip* mutants, there are mutants that do not show the pleiotropic phenotype displayed by *vip*s, molecular characterization of genes represented by these 'non-*vip*' mutants may identify factor(s) intrinsic to the vernalization mechanism. However, gene(s) that has been identified from other labs using the same mutant screening approach as mine may also be represented in our mutant collection [*e.g.*, *FRIGIDA LIKE1*, *ABA HYPERSENSITIVE1* (Michaels et al., 2004; Bezerra et al., 2004)]. Based on the phenotypic study of all of the mutants that have been recovered from my screen, some known *FLC* activators [e.g., EARLY IN SHORT DAYS4 and PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 (Reeves et al., 2002; Noh and Amasino, 2003)] are not likely represented in my mutant collection. Together with the fact that most of the *vip* mutants only have one allele, it is clear that my mutant screening was not saturated. Nevertheless, the successful recovery of four of the *vip* mutants (*vip3-1*, *vip4-1*, *vip5-1*, *vip6-1*, *vip6-2*) and mutants of several potential non-*VIP FLC* activators suggest that, although not being a saturated screen, the genetic approach that I used is effective to target *FLC* activators (or regulators).

Functionally redundant genes can be identified via analysis of gain-of-function mutations if they are sufficient to activate the pathways that they are involved in. Activation tagging, which has the potential to generate gain-of-function version of a particular gene (Hayashi *et al.*, 1992), could be a complementary strategy to identify *FLC* regulators. Based on the same mutant screening criteria as that for the *vip*s, mutants recovered from an activation tagging population likely represent genes that normally

suppress *FLC*, or flowering time genes that are capable of promoting flowering when overexpressed [e.g., *CONSTANS* (Onouchi et al., 2000)]. For the research area that I am involved in, the only gene that was identified so far using the activation tagging approach is the *AGAMOUS-LIKE20* (*AGL20*) [*a.k.a. SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Lee *et al.*, 2000; Samach et al., 2000)], which is a floral pathway integrator operating downstream of *FLC*. Considering the fact that a large number of *FLC* regulators have been isolated from "loss-of-function" mutagenesis, this gene discovery rate for the activation tagging approach is very low. The reason that put activation tagging approach in this apparently disadvantageous position for identifying *FLC* regulators might be due to the fact that most *FLC* regulators are not sufficient to regulate *FLC* by themselves [*e.g.*, *FVE*, *VIN3*, *VIP*s (Ausin *et al.*, 2004; Sung and Amasino, 2004; Zhang et al., 2003)]. Another reason could be the stringent requirement for the activation tagging cassette being targeted to the correct promoter regions to drive gene expression, making it difficult to identify novel genes from a relatively small population.

VIPs may represent a previously unknown transcription regulation mechanism in plants

VIPs were proposed to operate in a protein complex, in an analogous manner to the yeast Paf1C. The observation that VIP3, VIP4 and VIP6 physically interact suggests that VIPs may indeed be involved in a protein complex. To what extent this hypothetical plant complex resembles Paf1C remains to be further explored. VIP2 and VIP5 are homologues of Paf1 and Rtf1, respectively (Ek-Ramos and van Nocker, unpublished; He

et al., 2004; Oh et al., 2004), and their potential interactions with VIP3, VIP4, VIP6 remain to be tested. The interaction of VIP3, a protein that is not similar to any of the Paf1C components, with VIP4 and VIP6 suggests that the structure of Paf1C is not fully conserved in plants (Chapter 4). Most likely, Paf1C has recruited new components during evolution to 'handle' bigger genomes and more complicate chromatins. Molecular characterization of additional *VIP* genes and biochemical purification of the VIP complex offer two complementary approaches to resolve the structure and, more importantly, the function of the VIP complex.

The yeast Paf1C has been recently shown to be involved in generating a 'histone code' for transcriptionally active genes, by assisting histone H3K4 trimethylation (Ng et al., 2003a). Similarly, although VIPs are not likely involved in global histone methylation (Oh et al., 2004), VIP2 and VIP6 are apparently required for such histone modifications on *FLC* and *MAF1* chromatin (He et al., 2004). At this point, it is still not known what histone methyltransferase(s) (HMT) is recruited by VIPs. Some of the Paf1C dependent histone methylations require H2B ubiquitination (Ng et al., 2003b); if VIPs represent a plant Paf1C, are their functions also H2B ubiquitination dependent? Although histone ubiquitination has been shown to affect gene transcription in yeast, no such study has been documented for Arabidopsis or other plants. The Arabidopsis genome encodes homologues of Rad6 and Bre1 (yeast H2b ubiquitination enzymes) (Ludwig and van Nocker, unpublished), suggesting a potential of histone ubiquitination in this organism. A detailed characterization of these homologous proteins may help to elucidate their functions in plants.

Besides *FLC*, VIPs regulate a spectrum of other genes (Oh et al., 2004). How many of these genes are direct VIP targets is still unknown. Identification of the direct VIP target(s) is a crucial step for elucidating VIP function. In yeast, the VIP homologous proteins (Paf1C components) were shown to be physically associated with the elongating form the RNA polymerase II (Ng et al., 2003a) and distributed along open reading frames (Pokholok et al., 2002). If VIPs, like their yeast homologues, are also physically associated with the elongating form of RNA polymerase II, which can be tested by an immunocoprecipitation experiment with the appropriate antibody, it would be possible to identify genes that are directly targeted by the VIP mechanism through the ChIP-on-Chip approach (van Steensel and Henikoff, 2003).

How the VIP activation effect on *FLC* is counteracted by vernalization is also unanswered. It is tempting to think that vernalization may suppress *FLC* via negatively regulating VIPs. However, given the fact that *VIP2*, *VIP3*, *VIP4*, *VIP5*, *VIP6* mRNA and VIP3, VIP4, VIP6 protein levels remain unchanged after vernalizing cold (Ek-Ramos and van Nocker, unpublished; Zhang and van Nocker, 2002; Zhang et al., 2003; Oh et al., 2004), and the fact that vernalized wild-type plants do not phenocopy the *vip*s, it is unlikely that VIP function is completely annulled by vernalization. Two hypotheses have been raised to address this question. Briefly, vernalization may weaken, rather than compromise, VIP activity; or, alternatively, vernalization may change the *FLC* chromatin into a configuration that is repellent to the VIP mechanism (Oh et al., 2004). However, at least two additional scenarios cannot be ruled out to address the antagonistic effects of vernalization and VIPs on *FLC* expression. One scenario could be that vernalization may change the composition of the VIP complex, by removing a component that is crucial for

targeting or activating *FLC*. A comparison of the VIP complex components from vernalized and nonvernalized plants will test this hypothesis. The other scenario could be that VIPs, instead of functioning throughout the plant life cycle, function only in certain developmental stages (e.g., during gametogenesis or embryogenesis). In this case, vernalization and VIPs operate independently, in a temporally separated manner (i.e., vernalization does not directly affect VIPs' function). The observation that VIP4 protein is detectable only in the inflorescence tissue (Figure 2) would support this hypothesis. However, the presence of steadily detectable VIP3 and VIP6 in seedlings (young leaves) (Zhang et al., 2003; Oh et al., 2004) may argue against it. To further test this hypothesis, the temporal requirement of VIPs by plant to establish high *FLC* expression need to be determined. This could be explored by using a transgenic plant expressing the cloned *VIP*s, in an inducible (controllable) manner (e.g., VIP4-Glucocorticoid Receptor fusion).

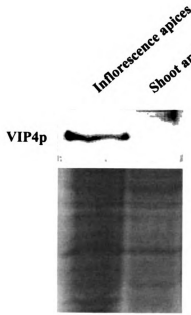


Figure 2. VIP4 protein is detected only in Arabidopsis inflorescence tissue. The tissue collection, protein extraction and western blotting were carried out essentially as described previously (Zhang and van Nocker, 2002; Zhang et al., 2003). The anti-VIP4 antibody was described in Chapter 4. A portion of representative SDS-PAGE gel, stained by Coomassie blue after blotting, is shown to indicate the relative quality and quantity of proteins in each lane.

Preliminary results

I am currently doing several experiments to further elucidate VIP function. These experiments are largely in progress. Here I present the data that was obtained, with a hope of helping future similar studies.

Identification of VIP4-interacting factors using a yeast two-hybrid approach.

An Arabidopsis meristem two-hybrid cDNA library was constructed using the HybriZAP-2.1 system (Stratagene, La Jolla, CA) following the supplier's instruction. The primary library contained 4.65 X 10^6 pfu (plaque forming units). The primary library was amplified once and converted to a plasmid (pAD-GAL4) library by *in vivo* mass excision following the manufacturer's protocol (Stratagene).

To construct a VIP4 bait for screening, a cDNA encoding the full length VIP4 protein was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using oligonucleotide primers EcoRI-VIP4 F 5'-AAGAATTCATGGTTAAAGGAGAA AAGAG-3' and SalI-VIP4 R 5'-AAGTCGACTTAATCTTCGTCACTGTCATC-3'. The PCR product was cloned into the EcoR I/Sal I site of the pBD-GAL4 vector and the construct was named as pBD-VIP4. The reading frame of the VIP4/pBD-GAL4 conjunction region was verified by sequencing.

For library screening, pBD-VIP4 was transformed into yeast YGR2 cells and the YGR2:pBD-VIP4 was subsequently transformed with cDNA library plasmid DNA mentioned above. The transformants were plated on selective medium [SD (synthetic dropout) lacking leucine, tryptophane and histidine (SD –L –W – H)] and incubated at 30 °C for six days. Any colonies that grew on the selective medium were streaked onto SD

–L –W –H media and assayed for β-galactosidase activity using the filter lift assay, following the supplier's instruction (Stratagene). Colonies that were capable of turning blue in the X-Gal assay were considered as potential positives. Plasmids from these clones were recovered using the Zymoprep yeast plasmid miniprep (Zymo Research, Orange, CA), transformed into E. coli strain DH5α, and purified. The purified plasmids were then transformed into yeast YGR2 either alone or with pBD-VIP4, pLamin C. The pLaminC, which expresses the BD of GAL4 and amino acids 67-230 of human lamin C, is used as a control for negative interactions with the identified VIP4 interacting clones. The transformants were plated onto the appropriate selective medium according to the provider's instruction (Stratagene). These transformants were subsequently evaluated for Lac Z activity and those that are capable of activating reporter gene only in the presence of pBD-VIP4 were considered as positives. The positives were sequenced using the oligonucleotide primer 5'-CCACTACAATGGATGATGTATA-3'.

A β-galactosidase assay was used to quantify interactions. Yeast was grown in selective medium (SD –Leu –Trp) overnight. 2ml of the overnight culture was inoculated into 8 ml YPD and the culture was grown to OD_{600} 0.6-0.8 at 30 °C. The OD_{600} was recorded. For each sample, 0.5 ml of culture was transferred into each of three 1.5 ml microcentrifuge tubes (thus three duplicate for each sample) and cells were pelleted by centrifuging. Cells were washed with 0.5 ml Buffer1 [100 mM HEPES, 155 mM NaCl, 2 mM L-Aspartate (hemi-Mg salt) (Sigma, St. Louis, MO), 1% BSA, 0.05% Tween 20, pH 7.25-7.3]. Cells were then pelleted and resuspend into 100 μl Buffer1 (concentration factor is 0.5/0.1=5). To break the cells, the cell suspensions were frozen in liquid N_2 for 1 min and then thawed in a 37 °C water bath for 1 min. After repeating the freeze-thaw

cycle 2 additional times, 0.7 ml of Buffer 2 [2.23 mM CPRG (chlorophenol red-β-D-galactopyranoside) in Buffer 1] was added to each tube and mix by votexing. The time of Buffer 2 addition was recorded. When the color of the samples started to turn red, 0.5 ml of 3.0 mM $ZnCl_2$ was added to each tube to stop color development. Samples were then centrifuged at 14,000 rpm for 1 min and the absorbance at 578 nm of the supernatant was measured. β-galactosidase activity was calculated using the formula: 1000 x $OD_{578}/(t \times V \times OD_{600})$; where: t=elapsed time (in min) of incubation; V=0.1 x concentration factor; $OD_{600}=A_{600}$ of 1 ml of culture when harvesting.

From screening of ~1.2 X 10^6 independent transformants, four clones, AD50, AD175, AD177 and AD341, showed weak interactions with VIP4 (Figure 3). All of these clones were annotated as encoding either unknown or putative proteins (Table 2). To evaluate the potential function of these genes in concert with *VIP4*, I did a RNA interference (RNAi) experiment to selectively degrade the transcripts of these genes. Approximately 600bp DNA fragment of each clone was amplified using primers described in Table 3 and cloned into the *Asc* I/*Swa* I and *Bam* HI/*Xba* I sites of vector pFGC5941, which carries the herbicide glufosinate resistant gene as plant selective marker. The plant transformation and transformant selection were essentially as described previously (Zhang and van Nocker, 2002). No discernible phenotype was observed after evaluating ~20 T1 individuals from each constructs (data not shown). However, it has not yet been determined if the function of the endogenous genes have been knocked out by RNAi. Thus, at this point, it is difficult to speculate the roles of these interacting proteins, if any, in the VIP mechanism.

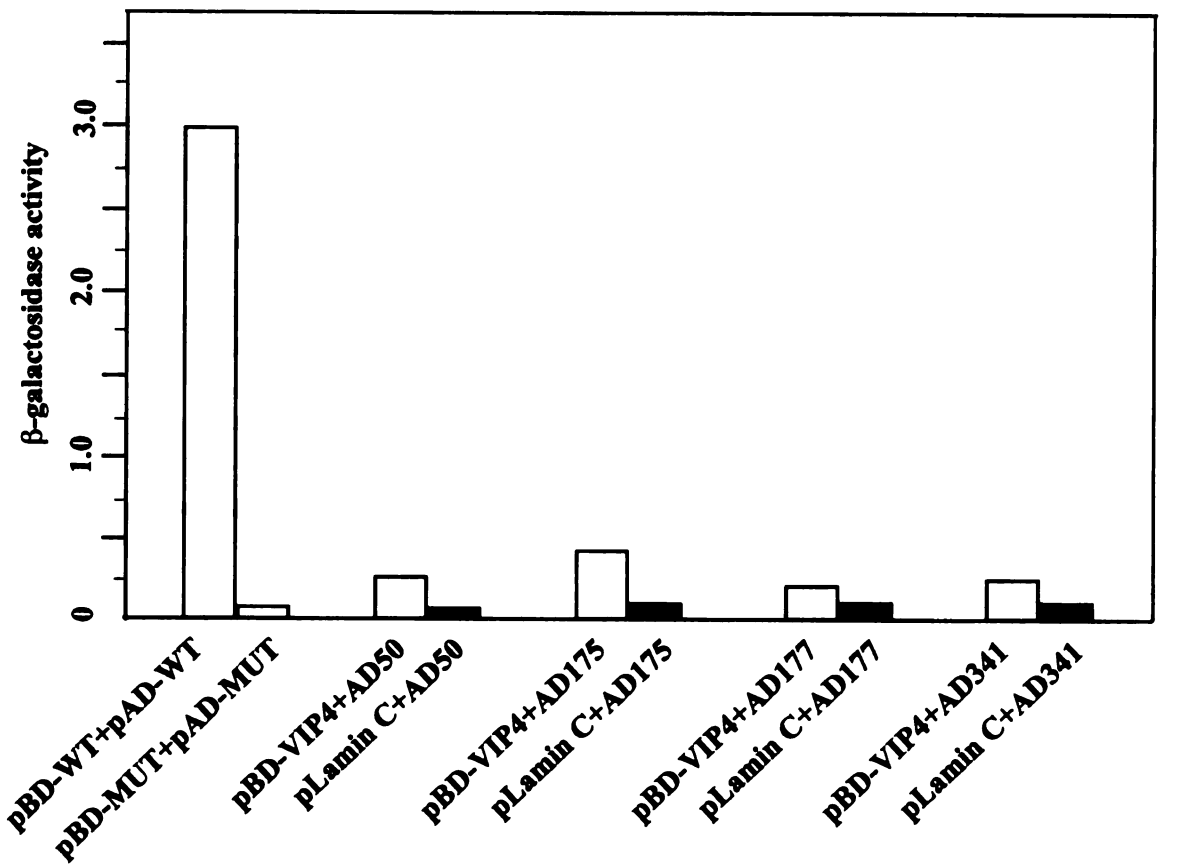


Figure 3. β-galactosidase activity assay of the four VIP4-interacting clones identified from the yeast two-hybrid screen. The plasmids pBD-WT, pAD-WT, pBD-MUT, pAD-MUT and pLaminC were provided by the manufacturer (Stratagene). The pBD-WT/pAD-WT and pBD-MUT/pAD-MUT were used as the positive control and negative control, respectively. The pLaminC, which expresses the BD of GAL4 and amino acids 67-230 of human lamin C, was used as a control for negative interactions with the identified VIP4-interacting clones. The data shows the mean value of three measurements of each sample. The standard deviations are too small to show in the figure.

Table 2. The VIP4 interacting proteins identified from the yeast two-hybrid screening. Annotations are from The Arabidopsis Information Resource (TAIR).

Clone	Protein	Annotation
AD50	At3g22790	Unknown protein
AD175	At4g28230	Putative protein
AD177	At2g39340	Unknown protein
AD341	At5g42520	Unknown protein

Table 3. The primer sequence for amplifying DNA fragment to construct plasmids for the RNAi experiment.

Name	Sequence
RNAi_AD50 F	AATCTAGAGGCGCGCCGTCAGATCCCTTGAGCAA
RNAi_AD50 R	AAGGATCCATTTAAATACTGTGATTCAACAGAGG
RNAi_AD175 F	AATCTAGAGGCGCGCCCTGAATATGACATCAATT
RNAi_AD175 R	AAGGATCCATTTAAATTTCCAGGAAGCTTAAAGA
RNAi_AD177 F	AATCTAGAGGCGCGCCGGGTCTGAAAGTGCCCCA
RNAi_AD177 R	AAGGATCCATTTAAATTTCTGTTTGGCTGTAATA
RNAi_AD341 F	AATCTAGAGGCGCGCCACCTTGGAATCTGCCAAA
RNAi_AD341 R	AAGGATCCATTTAAATTCATTTAATCGTAATGTA

A yeast two-hybrid screening approach was also used to screen VIP3 interacting proteins. However, no interacting clone was identified from a screening of ~1.0 X 10^6 independent transformants (data not shown).

Molecular mass study of the putative VIP complex.

Determination of the molecular mass of the putative VIP complex is a crucial step for identification of VIP components through a biochemical approach. To obtain enough inflorescence tissue for protein extraction, I used broccoli (*Brassica oleracea*) as the plant material. Broccoli is closely related to Arabidopsis and I have shown that anti-VIP3, anti-VIP4 and anti-VIP6 antibodies recognize appropriately sized proteins from broccoli extracts (Chapter 4, Figure 5).

Approximately 50 g of broccoli meristem tissues were homogenized in 100 ml extraction buffer [50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10^{-4} mM pepstatin A] using a Waring blender (Fisher Scientific, Pittsburgh, PA). The homogenate was filtered through two layers of cheesecloth and four layers of mirocloth (Calbiochem, La Jolla, CA) and then centrifuged at 39,000 Xg for 30 min. The supernatant was precipitated with 40% $(NH_4)_2SO_4$ at ice temperatures. After centrifugation at 20,000 X g, the pellet was resuspended into 10 ml of column running buffer [Tris-HCl (pH 7.5) 50 mM, NaCl 150 mM, glycerol 10%]. For gel filtration, 5ml samples were applied to a Sephacryl S-400 (Amersham Biosciences, Piscataway, NJ) column pre-equilibrated with the running buffer. The column was run at a speed of 10 ml/hr and 8ml fractions were collected. All gel filtration procedures were performed at 4 °C. To determine fractions containing

VIPs, 20 µl of each fraction was separated by SDS-PAGE and blotted onto PVDF membranes (Bio-Rad, Richmond, CA). The blots were probed using anti-VIP3 antibody following a procedure described previously (Zhang et al., 2003). The reason to choose anti-VIP3 antibody is that this antibody recognizes BoVIP3 strongly (Chapter 4, Figure 5).

Anti-VIP3 immunoreactive protein species were present in all tested fractions (Figure 4), with a molecular mass ranging from ~40 kDa to ~2 MDa. This is not likely due to a failure of protein separation, as judged from comparing the protein profiles of each fraction on SDS-PAGE gel (data not shown). One possibility could be that the Sephacryl matrix has a strong tendency to bind Bo-VIP3. Other possibilities could be that the association of VIP3 with the putative complex is not stable under the chromatography conditions, or that VIP3 is involved in different types of complexes with a wide range of molecular mass (e.g., bound with genomic DNA).

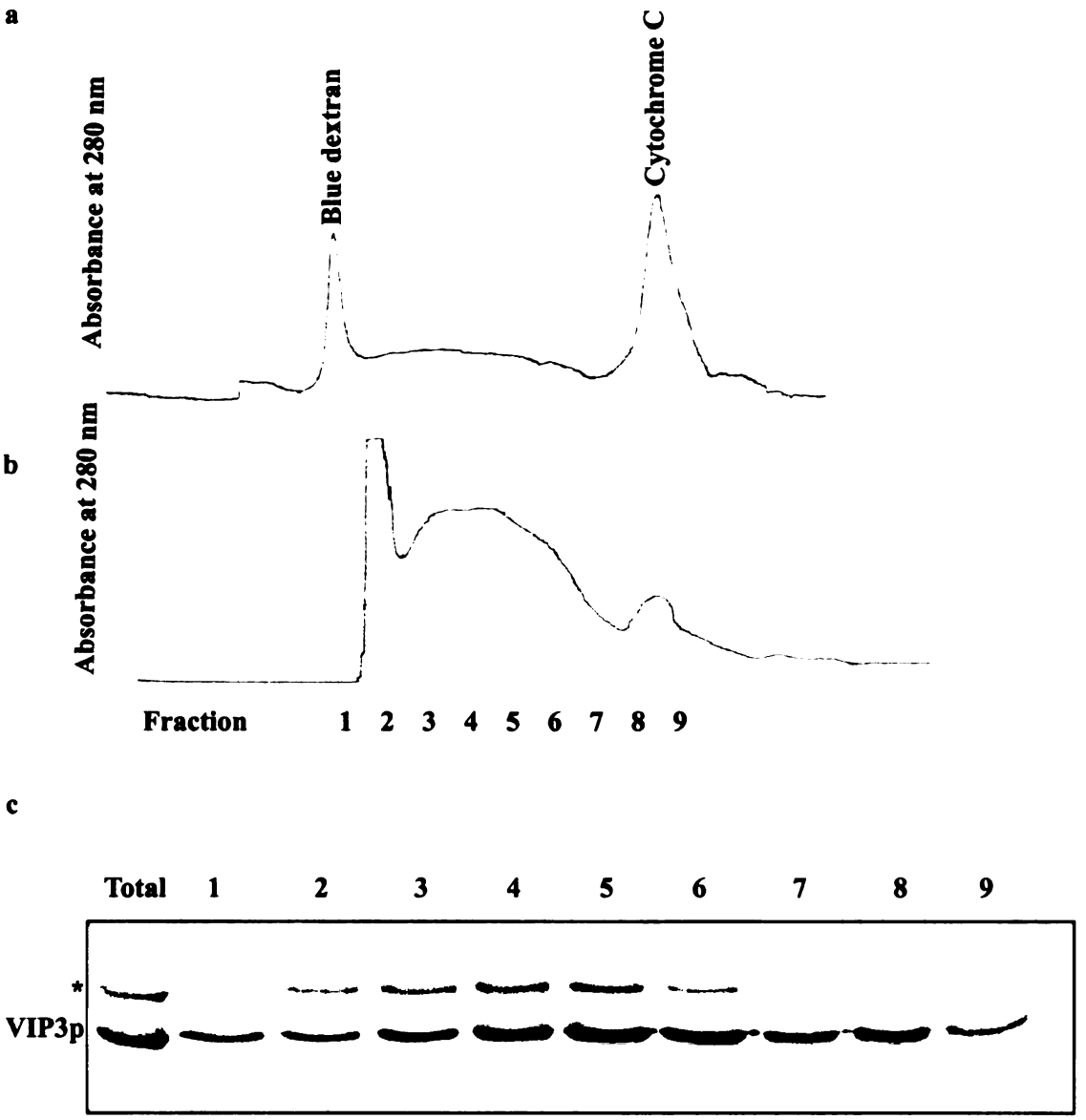
Epistatic analysis of VIP4 and autonomous pathway genes.

Although previous study suggested that VIP4 might operate in a mechanism independent of the autonomous flowering promotion pathway, the result was obtained from molecular and genetic epistatic analysis of *VIP4* and a representative autonomous pathway gene, *LD*. We now know that autonomous pathway genes promote flowering through distinct mechanisms and these genes do not comprise a linear pathway. A detailed epistatic analysis between *VIP4* and all autonomous pathway genes is necessary to clearly elucidate the interactions, if any, between VIPs and the autonomous pathway.

To address this question, I crossed *vip4-2*, which is resistant to the herbicide

Figure 4. *B. oleracea* VIP3 is detected in every fraction with a molecular weight range of 40 kDa ~ 2 MDa in a gel infiltration column chromatography experiment.

(a) Calibration of the gel infiltration column. The retention time of the standard molecular marker, Blue dextran and Cytochrome C, are designated.
(b) Gel infiltration of the *B. oleracea* total protein. The retention time of fraction 1 through fraction 9 are designated. The elution curve is aligned to the column calibration curve shown in panel a.
(c) 20 µl samples from each of the 8 ml fractions were separated on SDS-PAGE and blotted onto PVDF membrane for western blotting analysis. Western blotting analysis and the anti-VIP3 antibody were as described previously (Zhang et al., 2003). The numbers indicate each of the 8 ml fractions. The '*' indicates an unrelated, VIP3-immunoreactive protein species present in broccoli total protein extracts and some of the fractions.


a
Absorbance at 280 nm
Blue dextran
Cytochrome C
b
Absorbance at 280 nm
Fraction 1 2 3 4 5 6 7 8 9
c
Total 1 2 3 4 5 6 7 8 9
*
VIP3p

glufosinate, with autonomous pathway mutants *ld*, *fld*, *fve*, *fpa*, *fy* and *fca*. All of these autonomous pathway mutants are in the Col-0 background. The *ld* (*ld-1*) mutant is as described previously (Zhang and van Nocker, 2002). The *fld* (SALK_075401), *fve* (SALK_013789), *fpa* (SALK_085616) and *fy* (SALK_053604) mutants are SALK T-DNA lines, obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University (Columbus, Ohio). The *fca* mutant (*fca-9*) was a generous gift from C. Dean (John Innes Centre, Norwich, UK). Double mutants were constructed using standard genetic techniques. The primer sequences of PCR/cleaved amplified polymorphic sequence (CAPS) markers for *ld*, *fca*, *fpa*, *fy*, and *fld* mutants are listed in Table 4. The PCR marker provided by the SALK institute for the *fve* mutant did not give consistant results. Thus, the *fve vip4-2* double mutant was obtained by selfing an *fve/fve vip4-2/VIP4* plant. Flowering time was measured under long-day condition.

I have obtained all the double mutants. However, I have yet to analyze the flowering time.

Physical interaction of VIP4 and the elongating form of RNA polymerase II.

In yeast, Paf1C components are found to be physically associated with the elongating form of RNA polymerase II (Pol II) (i.e., the CTD Ser2-phosphorylated form) (Ng et al., 2003a). If VIPs also interact with the elongating form of RNA Pol II, it will be feasible to identify VIPs target genes through the ChIP-on-Chip approach using microarrays currently available to Arabidopsis researchers (van Steensel and Henikoff, 2003).

I conducted an immunocoprecipitation (CoIP) experiment to test the potential

Table 4. The primers for the PCR/cleaved amplified polymorphic sequence (CAPS) markers of the corresponding autonomous pathway mutants. For PCR marker, an N/A is put in the "Enzyme" column; for CAPS markers, a restriction enzyme is listed in the "Enzyme" column. The product size for *fld*, *fpa*, *fy* mutants indicate the fragments amplified from the wild-type alleles, no fragment will be amplified from the mutant alleles.

Mutant	Primer	Enzyme	Product size
ld-1	ld-1 MseIF: GCTGCGTAGCTTTCATCAATGCCA ld-1 MseIR: GAATATCTTCCTGTTACGACACG	Mse I	*LD*: 386 bp *ld-1*: 333 bp
fld	SALK_075401 LP: CGGATCCATCAAATTTGTTCCC SALK_075401 RP: GCAGGAGATTTTGGGTCTGGA	N/A	883 bp
fpa	SALK_085616 LP: CTTCCTGAAGCATCGAGCCAA SALK_085616 RP: GCCGGAGACGACAGAGTCAGA	N/A	973 bp
fy	SALK_053604 LP: CATGCCACCTTGCATTCCCTA SALK_053604 RP: GGTTCGAAATGGACCTTGGCT	N/A	929 bp
fca-9	RLFCA9: TCTTTGGCTCAGCAAACC GSO379: TGTTGAGATGGTGAAACTGTG	Sty I	*FCA*: 250 bp *fca-9*: 210 bp

physical interaction between VIP4 and elongating form of RNA Pol II. The antibody recognizing the elongating form of RNA Pol II was obtained from Covance Research Products (Denver, PA). This antibody recognizes a single protein species with the expected molecular weight for the Arabidopsis RNA Pol II (~240 kDa) from inflorescence extract, but not from the leaf extract (Figure 5). For immunoprecipitation experiments, the protein was extracted using the extraction buffer essentially as described (Oh et al., 2004), but with an addition of phosphatase inhibitors (1 mM $Na_4P_2O_7$, 10 mM NaF, 100 μM β-glycerolphosphate, 1mM Na_3VO_4). The immunocoprecipitation procedure was performed as previously described (Oh et al., 2004).

From the CoIP experiment, the anti-VIP4 IgG did not pull down the elongating form of RNA Pol II (Figure 6 top). The absence of Pol II Ser2-P in the immunoprecipitates is not likely due to a failure of the CoIP, because, as a positive control, I successfully pulled down the VIP6 with the anti-VIP4 IgG (Figure 6 bottom panel). Also, the absence of Pol II Ser2-P in the immunoprecipitates is not likely due to possible de-phosphorylation of the Pol II Ser2-P (Figure 6 top panel, lane "de-phos control"). These results indicate that VIP4 probably does not physically interact with the elongating form of RNA Pol II in vivo. However, we cannot rule out the possibility that VIP4 and Pol II Ser2-P interact weakly or transiently.

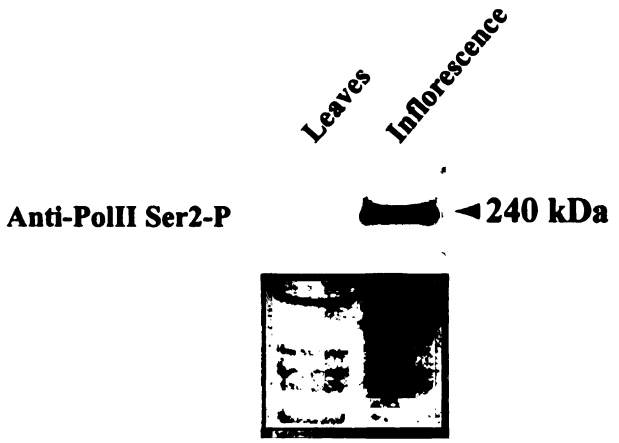


Figure 5. Detection of the elongating form of RNA polymerase II in Arabidopsis.
The designated antibody recognizes a single protein species with a predicted molecular weight from Arabidopsis inflorescence tissue extract. Only a marginally detectable amount of such protein species is present in leaf extracts.
A portion of the corresponding SDS-PAGE gel, stained by Coomassie blue after blotting, is shown to indicate the relative quality and quantity of proteins in each lane.

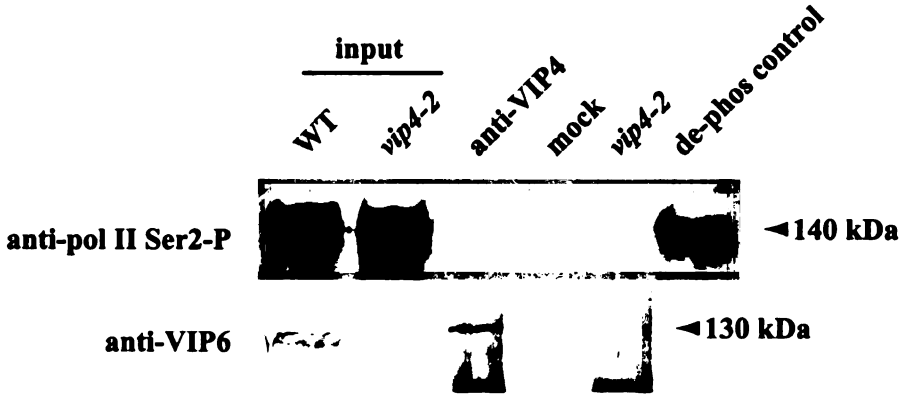


Figure 6. VIP4 probably does not physically interact with elongating form of RNA polymerase II in vivo.

Total protein from wild-type and *vip4-2* mutant inflorescence apices (two lanes at left in each panel) was subjected to immunoprecipitation using anti-VIP4 IgGs.

(top) Immunoprecipitates were analyzed by protein gel blotting using anti-Pol II Ser2-P antibody. No immunoreactive protein was detected. The "de-phos control" lane is a sample of WT total protein incubated along with the immunoprecipitation samples, served as a control of potential de-phosphorylation of the RNA pol II Ser2-P that might cause the missing of RNA pol II Ser2-P signal.
(bottom) Immunoprecipitates from were subjected to protein gel blotting using anti-VIP6 antibody. The successful pull-down of VIP6 indicates that the absence of RNA pol II Ser2-P is not due to a failure of immunocoprecipitation.

References

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004) Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat. Genet. 36: 162-166.

Bezerra, I.C., Michaels, S.D., Schomburg, F.M., and Amasino, R.M. (2004) Lesions in the mRNA cap-binding gene *ABA HYPERSENSITIVE1* suppress *FRIGIDA*-mediated delayed flowering in Arabidopsis. Plant J. 40: 112-119.

Gendall, A.R., Levy, Y.Y, Wilson, A., and Dean, C. (2001) The *VERNALIZATION2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell 107: 525-535.

Hayashi, H., Czaja, I., Lubenow, H., Schell, J., and Walden, R. (1992) Activation of a plant gene by T-DNA tagging: Auxin-independent growth *in vitro*. Science 258: 1350-1353.

He, Y., Doyle, M. R., and Amasino, R. M. (2004) PAF1-complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. Genes Dev. 18: 2274-2284.

Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M., and Lee, I. (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev. 14: 2366-2376.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R. and Dean, C. (2002) Multiple Roles of Arabidopsis VRN1 in Vernalization and Flowering Time Control. Science 297: 243-246.

Michaels, S.D. and Amasino, R.M. (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., Bezerra, I.C., and Amasino, R.M. (2004) *FRIGIDA*-related genes are required for the winter-annual habit in Arabidopsis. Proc Natl Acad Sci U.S.A. 101: 3281-3285.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2003a). Targeted recruitment of Set1 histone methylase by elongating PolII provides a localized mark and memory of recent transcriptional activity. Mol. Cell 11: 709-719.

Ng, H.H., Dole, S., and Struhl, K. (2003b) The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. J Biol. Chem. 278: 33625-33628.

Noh, Y.S., and Amasino, R.M. (2003). *PIE1*, an ISWI family gene, is required for *FLC* activation and floral repression in Arabidopsis. Plant Cell 15: 1671-1682.

Oh, S., Zhang, H., Ludwig, P., and van Nocker, S. (2004) A mechanism related to the yeast transcriptional regulator Paf1C is required for expression of the Arabidopsis *FLC/MAF* MADS-box gene family. Plant Cell 16: 2940-2953.

Onouchi, H., Igeno, M.I., Perilleux, C., Graves, K., and Coupland, G. (2000) Mutagenesis of plants overexpressing *CONSTANS* demonstrates novel interactions among Arabidopsis flowering-time genes. Plant Cell 12: 885-900.

Pokholok, D.K., Hannett, N.M., and Young, R.A. (2002) Exchange of RNA polymerase II initiation and elongation factors during gene expression *in vivo*. Mol. Cell 9: 799-809.

Reeves, P.H., Murtas, G., Dash, S., and Coupland, G. (2002) *early in short days4*, a mutation in Arabidopsis that causes early flowering and reduces the mRNA abundance of the floral repressor *FLC*. Development 129: 5349-5361.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science 288: 1613-1616.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999) The *FLF* MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell 11: 445-458.

Sung, S., and Amasino, R.M. (2004) Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature 427: 159-164.

van Steensel, B. and Henikoff, S. (2003) Epigenomic profiling using microarrays. Biotechniques 35: 346-357

Zhang, H. and van Nocker, S. (2002) The *VERNALIZATION INDEPENDENCE4* gene encodes a novel regulator of *FLOWERING LOCUS C*. Plant J. 31: 663-673.

Zhang, H., Ransom, C., Ludwig, P., and van Nocker, S. (2003) Genetic analysis of early-flowering mutants in Arabidopsis defines a class of pleiotropic developmental regulator required for activity of the flowering-time switch *FLOWERING LOCUS C*. Genetics 164: 347-358.