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INVITED REVIEW

Memories of winter: vernalization and the competence to flower

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ABSTRACT

The promotion of flowering in response to a prolonged exposure to cold temperatures (i.e. winter) is a useful adaptation for plant species that flower in the spring. This promotion is known as vernalization and results in a permanent memory of cold exposure. While the physiology of vernalization has been extensively studied in many species, the molecular mechanism of vernalization remains largely unknown. Recent studies, however, have revealed some of the molecular events that create the requirement for vernalization. In Arabidopsis, naturally occurring late-flowering ecotypes and plants containing late-flowering mutations in the autonomous floral-promotion pathway are relatively late flowering unless cold treated. The vernalization requirement of these late-flowering ecotypes and autonomous-pathway mutants is largely created by an upregulation of the floral inhibitor *FLOWERING LOCUS C (FLC)*. After cold treatment, as imbibed seeds or young seedlings, *FLC* transcript levels are downregulated and remain low for the remainder of the plant's life, but return to high levels in the next generation. Plants containing a constitutively expressed *35S:FLC* construct remain late flowering after cold treatment, indicating that *FLC* levels must be downregulated for vernalization to be effective. Thus the epigenetic downregulation of *FLC* appears to be a major target of the vernalization pathway and provides a molecular marker of the vernalized state.

Key-words: biennial; cold perception; epigenetic; flowering; *FLOWERING LOCUS C*; *FRIGIDA*; vernalization

INTRODUCTION

The change from vegetative to reproductive development marks a major transition in the plant life cycle. Meristems that had been producing vegetative structures, such as leaves, switch to producing flowers. In many plant species the transition of the meristem from vegetative to reproductive development is irreversible; thus, the proper timing of this transition is very important for successful reproduction. Accordingly, plants have evolved mechanisms to control flowering time in response to environmental cues, and therefore co-ordinate flowering with particular seasons. Two common environmental cues that can affect flowering time are day-length (photoperiod) and cold. Much is now known about the molecular basis of day-length perception: light receptors such as phytochromes and cryptochromes monitor day-length, components of the downstream signal transduction pathway have been identified in Arabidopsis such as the *CONSTANS* transcription factor, and components have also been identified that may interface with the circadian clock such as *GIGANTEA* and the myb-like factors *CIRCADIAN CLOCK ASSOCIATED 1* and *LHY* (reviewed in Koornneef *et al*. 1998; Simpson, Gendall & Dean 1999). In contrast, little is currently known of the molecular basis of the promotion of flowering by cold.

The promotion of flowering in response to a prolonged exposure to cold temperatures (i.e. winter) is a useful adaptation for plant species that flower in the spring. This promotion is known as vernalization. Chouard (1960). defined vernalization as 'The acquisition or acceleration of the ability to flower by a chilling treatment.' Thus, a vernalizing cold treatment does not initiate flower primordia directly, but creates the capacity for subsequent flowering. Vernalization does not refer to the breaking of dormancy by cold, such as the release of pre-formed floral buds after chilling or the promotion of seed germination by cold (stratification). The vernalization response can be facultative or obligate. Winter annuals, for example, have a facultative vernalization response; cold exposure is not required for flowering, but flowering will occur more rapidly after cold treatment. Biennials, in contrast, have an obligate requirement for cold treatment and thus cannot flower without prior cold exposure.

PHYSIOLOGY OF VERNALIZATION

A number of excellent reviews have been written on the physiology of vernalization (Bernier, Kinet & Sachs 1981; Chouard 1960; Lang 1965); thus, only a brief summary is presented here. Among plants in which flowering is promoted by cold, the range of effective temperatures is generally 1–7 °C. Some cereals, however, can be vernalized at temperatures as low as – 6 °C (Bernier *et al*. 1981) and in

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certain plants native to warmer regions, such as olive, temperatures as high as 13 °C are effective (Hackett & Hartmann 1967). The duration of cold treatment required to promote flowering also varies, with 1–3 months of cold being typical. In some species such as celery, as little as 8 days of cold can cause a substantial acceleration of flowering; however, greater than 1 month of cold treatment is required for maximal promotion of flowering (Thompson 1944). The consistency of the cold treatment can also impact the effectiveness of vernalization and, like the temperature and duration of cold treatment, the optimum is species dependent. For example, in rye warm-temperature breaks during the cold treatment disrupt vernalization (Purvis & Gregory 1952), while celery is insensitive to breaks in cold treatment (Thompson 1944).

Metabolic activity is required for vernalization. Thus, dry seeds cannot be vernalized, although imbibed seeds of many species are responsive. In fact, seeds of many cereals can be imbibed with an amount of water that is sufficient for vernalization, but insufficient for germination (Purvis 1961). This discovery was agriculturally important; preventing germination allowed vernalized seeds to be sown with standard planting equipment. In some species, such as rye, embryos can even be vernalized prior to seed desiccation (Chouard 1960). Other plants, however, cannot be vernalized as imbibed seeds or young seedlings but rather must reach a critical age or developmental stage before vernalization can occur. One example is biennial *Hyocyamus niger*, which is insensitive to cold treatment before 10 days of age under normal growing conditions (Lang 1986).

Localized cooling experiments and grafting experiments have shown that in intact plants the shoot apex must be exposed to cold for vernalization to occur (Lang 1965). This is consistent with vernalization causing the apical meristem to acquire competence to flower. Other experiments, however, indicate that other mitotically active tissues can become vernalized. Such experiments are possible because of the ability of somatic cells to regenerate into complete plants. Wellensiek, for example, working with cuttings of cold-treated *Lunaria biennis*, showed that leaves that were fully grown at the beginning of the cold treatment regenerated only into plants that remained vegetative, whereas leaves and roots that were growing at the start of the cold treatment regenerated into flowering shoots (Wellensiek 1962; Wellensiek 1964). This led Wellensiek (1964) to propose that mitotic activity is required for vernalization. Similar experiments performed in *Thlaspi arvense*, however, showed that even leaves that were fully expanded at the beginning of the cold treatment regenerated into flowering plants (Metzger 1988). Although the results from these two species appear to differ, two things are clear from these experiments: cells in organs other than the shoot apex can become vernalized, and not all cells can become vernalized (e.g. cells from fully grown leaves of *Lunaria*). Whether or not cell division is required for cells to become vernalized is an open question and the answer may be species specific. It is possibile that some localized cell division is taking place in fully expanded *Thlaspi* leaves. Also, even in the absence of cell division, DNA replication may still occur. Studies in Arabidopsis, a relative of *Thlaspi*, have revealed extensive endoreduplication in leaves (Galbraith, Harkins & Knapp 1991). This raises the possibility that it may be DNA replication that is required for acquisition of the vernalized state rather than mitosis *per se*. Indeed, DNA replication is often required to reprogramme gene expression during development in a broad range of organisms (e.g. Miller & Nasmyth 1984).

In most species the effect of a vernalizing cold treatment can be partially or totally eliminated by several days of heat treatment, typically 30–40 °C (devernalization) (Bernier *et al*. 1981). The heat treatment, however, must be applied immediately after cold treatment; after several days at normal growth temperatures devernalization is ineffective. Furthermore, the ability of heat treatment to 'devernalize' declines with increased duration of cold treatment. It is interesting to note that in some plants, such as Arabidopsis and certain varieties of Chrysanthemum, devernalization is only effective if the plants have been cold treated in darkness (reviewed in Bernier *et al*. 1981). This may reflect a requirement for a threshold level of metabolic activity or cell division in order for the vernalized state to become 'fixed'.

Once the vernalized state has been achieved, it is stable throughout subsequent mitotic divisions. This is apparent from the ability of cold-treated leaf or root cells to regenerate into vernalized plants as described above. The stability of the vernalized state was first demonstrated in studies of plants that have an obligate requirement for both cold and inductive photoperiods such as biennial *H. niger*, which has an obligate requirement for cold followed by long days before flowering can occur. Vernalized biennial *H. niger* plants remain vegetative as long as they are maintained under short days, but flower readily when transferred to long days even if grown in short days for periods of time during which all of the leaves and leaf primordia that were present during cold treatment have abscized (Lang 1965). Only after a 300 day delay between cold treatment and inductive photoperiods was there any evidence of a decline in the vernalized state (Lang 1965). The vernalized state is not, however, passed to the next generation. Progeny derived from vernalized plants must be re-exposed to cold to become vernalized.

MOLECULAR BASIS OF VERNALIZATION

The mitotic stability of the vernalized state and the resetting of this state in the next generation is consistent with a model of vernalization involving epigenetic switching of gene expression that is reminiscent of genomic imprinting in animals. Genomic imprinting is a term used to describe the phenomenon of expression of only the maternal or paternal copy of certain genes in progeny (Simon *et al*. 1999; Tilghman 1999). The system is reset as these genes pass to the next generation. For example, a gene for which only the maternal copy is active becomes inactive when a male passes that allele to progeny (Tilghman 1999). Recent work

on the *MEDEA* locus indicates that a similar system may operate in plants (Grossniklaus *et al*. 1998; Russinova & de Vries 2000). In some cases epigenetic changes in gene expression such as imprinting have been associated with changes in DNA methylation (Tilghman 1999).

It has been proposed that vernalization may involve changes in the pattern of DNA methylation (Dennis *et al*. 1997; Finnegan *et al*. 1998). In support of this model, the level of DNA methylation is reduced in *Nicotiana plumbaginifolia* cell cultures (Burn *et al*. 1993a) and Arabidopsis plants after cold treatment (Finnegan *et al*. 1998). Furthermore, in Arabidopsis lines that exhibit earlier flowering after vernalization, earlier flowering is also observed when DNA methylation levels are reduced by expression of an antisense methyltransferase construct (Finnegan *et al*. 1998) or after exposure to the demethylating agent 5-azacytidine (Burn *et al*. 1993a). Plants that have reduced methylation levels, however, show a number of pleiotropic effects including ectopic expression of floral organ-identity genes such as *AGAMOUS* (Finnegan, Peacock & Dennis 1996) which are known to induce flowering when ectopically expressed (Mizukami & Ma 1997). Thus it is unclear if the early flowering phenotype of lines with reduced levels of DNA methylation is due to the activation of the vernalization pathway or inappropriate expression of genes such as *AGAMOUS*. Furthermore, as noted by Finnegan *et al*. (1998), the promotive effects of vernalization and demethylation on flowering are additive which is consistent with vernalization and demethylation acting on separate flowering pathways (or with demethylation not fully activating the vernalization pathway). To date there have not been any reports identifying specific genes that are differentially methylated after vernalization. In general, epigenetic changes of gene transcription rates are likely to involve an alteration in chromatin structure, and methylation changes are one mechanism that can contribute to altering chromatin structure. There are examples of imprinting in animals that do not appear to involve changes in methylation (Caspary *et al*. 1998) and there are many examples in Drosophila, an organism which does not have DNA methylation, of epigenetic switches in gene expression due to other systems of chromatin remodelling that do not involve methylation (Cavalli & Paro 1999). Thus changing patterns of DNA methylation may contribute to the epigenetic changes in gene expression that are likely to be responsible for the vernalized state, but more research is required to establish the molecular basis of this phenomenon.

In many species the plant hormone gibberellin (GA) plays a role in the regulation of flowering. For example, GA biosynthesis or response mutants often exhibit delayed flowering (Pharis & King 1985). Furthermore, in many long-day and cold-responsive plant species, such as biennial *H. niger*, exogenous GA application can cause flowering in the absence of inductive photoperiods or cold treatment (Zeevaart 1983). Might an increase in GA biosynthesis or an alteration of GA metabolism be a mechanism for the promotion of flowering by vernalization? Other observations indicate that GA acts to promote flowering more generally and may not be directly involved in vernalization. For example, in Arabidopsis applied GAs are effective in reducing the time to flowering in wild-type strains as well as late-flowering mutants. However, the promotion of flowering by applied GA does not mimic vernalization, because the time to flowering is reduced similarly in vernalization-sensitive and vernalization-insensitive late-flowering mutants (Chandler & Dean 1994). Also, late-flowering, vernalization-responsive strains of Arabidopsis respond normally to cold treatment in the presence of the *ga1-3* mutation (Michaels & Amasino 1999b). The *ga1-3* allele is a deletion in *KAURENE SYNTHASE*, a gene encoding an enzyme that catalyses the first committed step of GA biosynthesis (Sun, Goodman & Ausubel 1992). Thus GA may not be directly involved in the vernalization pathway.

It is interesting to note that there are situations in which the cold requirement of a biennial plant can be eliminated by grafting. Non-cold-treated biennial *H. niger* receptor plants have been induced to flower after being grafted to annual or cold-treated biennial varieties of the same or different species (Lang 1965). In most cases the graft partners that caused flowering in non-cold-treated *H. niger* receptors were grown under conditions that promote flowering. An exception, however, was that grafting the Maryland Mammoth mutant of tobacco under day-length conditions that are either inductive (short days) or non-inductive (long days) for Maryland Mammoth caused flowering of *H. niger* in the absence of cold treatment. One interpretation of all of these grafting results is that there may exist a transmissible vernalization hormone (vernalin) (Lang 1965), and that vernalin is produced not only in cold-requiring plants after vernalization but also is produced constitutively in some plants that do not have a vernalization requirement, such as Maryland Mammoth. Another possibility is that flowering in *H. niger* may be triggered by GAs supplied by the graft partner. Most of the graft donors that caused flowering in non-cold-treated *H. niger* were grown under inductive conditions, and GAs levels often increase in plants in inductive long-day photoperiods (e.g. Zeevaart & Gage 1993; Hedden & Kamiya 1997). As discussed above, applied GAs cause flowering in *H. niger* in the absence of both cold and inductive photoperiod. Similarly, grafts to Maryland Mammoth also cause flowering in *H. niger* in the absence of both cold and inductive photoperiod for *H. niger*. Therefore one possibility is that, regardless of photoperiod, sufficient graft-transmissible GAs may be produced by Maryland Mammoth to cause flowering in *H. niger.*

GENETICS OF VERNALIZATION

Many species of plants exist as both annual and biennial varieties and crosses between such varieties have been used to genetically identify loci that are responsible for the annual or biennial habit. Biennialism is usually conferred by a small number of loci, which may be dominant or recessive depending upon the species. *H. niger* was one of the first plants in which the genetics of the biennial habit was

studied. Correns showed that the difference in flowering behaviour between annual and biennial strains is due to a single dominant locus conferring the biennial phenotype (as reviewed in Lang 1986). In sugar beet, however, the biennial habit is conferred by a single recessive locus (Abegg 1936). Genetic analyses of vernalization in spring and winter varieties of diploid wheat and barley have revealed two genes that act antagonistically. Dominant alleles of *Vrn-Am1* from wheat and *Vrn-H1* from barley reduce the requirement for vernalization and promote a spring growth habit, while dominant alleles of *Vrn-Am2* and *Vrn-H2* increase the vernalization requirement and promote the winter growth habit (Tranquilli & Dubcovsky 2000).

Arabidopsis has been used as a model system to study numerous aspects of plant development including flowering time. Flowering in Arabidopsis is promoted by long days and by vernalization in a facultative manner (Napp-Zinn 1969). The dispensable nature of the promotion of flowering by long days and vernalization makes Arabidopsis an attractive genetic system in which to study these processes because plants containing mutations in these pathways will eventually flower and thus facilitate genetic analyses. A large number of loci have been identified that when mutated alter the timing of flowering (Koornneef *et al*. 1998). Genes identified by late-flowering mutations are likely to promote flowering; i.e. such mutations result in the loss of a promoter of flowering. Likewise, early flowering mutations identify genes whose wild-type function is to delay flowering.

In addition to mutational analysis, naturally occurring variation in flowering time between ecotypes of Arabidopsis has been used to identify genes that affect flowering time. In contrast to the rapid-cycling summer-annual strains favoured for most aspects of Arabidopsis research, many ecotypes of Arabidopsis are extremely late flowering unless vernalized (i.e. these ecotypes behave as winter annuals). Crosses between early-flowering summer-annual laboratory strains and winter-annual strains have shown that variation at one or both of two loci, *FLOWERING LOCUS C (FLC)* and *FRIGIDA (FRI),* is responsible for the late-flowering, vernalization-responsive habit of the winter annuals (Napp-Zinn 1987; Burn *et al*. 1993b; Lee, Bleecker & Amasino 1993;Clarke & Dean 1994; Koornneef *et al*. 1994; Lee *et al*. 1994). *FLC* and *FRI* interact synergistically to delay flowering; a loss-of-function mutation in either gene results in a complete elimination of the late-flowering phenotype (S. D. Michaels and R. M. Amasino, unpublished results).

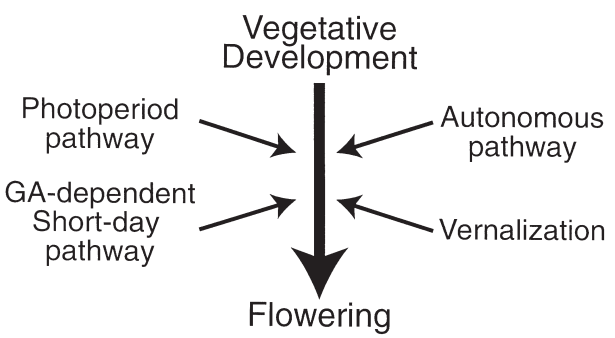

Figure 1. Circuitry of flowering-time control in Arabidopsis.

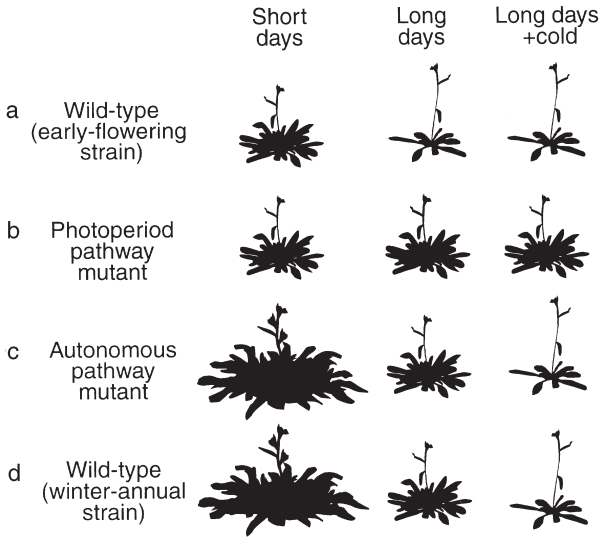

Figure 2. Photoperiod and vernalization responsiveness of Arabidopsis ecotypes and late-flowering mutants. Early flowering wild-type strains flower more rapidly in response to long days. Late-flowering mutants obtained from early flowering parents (a) exhibit two classes of responses. Photoperiod-pathway mutants (b) flower similarly to wild type under SD, but their flowering is not accelerated by long days or by vernalization. Autonomous-pathway mutants (c) are later flowering than wild type under short days and long days, but their late-flowering phenotype can be eliminated by vernalization. The flowering behaviour of winter-annual strains (d) is similar to autonomous-pathway mutants.

Genetic and physiological studies suggest that there are at least four pathways that promote flowering in Arabidopsis: a photoperiod pathway, a GA-dependent pathway (Wilson *et al*. 1992; Blazquez *et al*. 1998), an autonomous or constitutive pathway, and a vernalization pathway (Fig. 1) (Koornneef *et al*. 1998; Simpson *et al*. 1999). Evidence for the photoperiod and autonomous pathways was first obtained from the analysis of late-flowering mutants. Mutants in one group of genes, *CONSTANS (CO), FD, FE, FHA, FT, FWA*, and *GIGANTEA* (*GI*), are delayed in flowering under long days but not in short days (i.e. these mutants are 'blind' to photoperiod; Fig. 2a,b). Thus these genes are thought to act in a photoperiod-responsive pathway that promotes flowering in response to long days. A second group of genes, *FCA, FLOWERING LOCUS D (FLD), FPA, FVE, FY,* and *LUMINIDEPENDENS (LD),* is referred to in the literature as promoting flowering in an 'autonomous' or photoperiod-independent manner because plants with mutations in these genes are later flowering than wild type under both LD and SD (i.e. the mutants exhibit a photoperiod response but flowering is

much delayed compared with the early flowering, wild-type strain from which the mutants were derived; Fig. 2a,c). The late-flowering phenotype of autonomous-pathway mutants is readily eliminated by vernalization, in contrast to photoperiod-pathway mutants that are only slightly responsive to vernalization periods that effectively eliminate the phenotype of autonomous-pathway mutants.

It is important to note that the flowering behaviour of autonomous-pathway mutants derived from early flowering strains is similar to the late-flowering, vernalization-responsive phenotype of wild-type winter-annual strains of Arabidopsis containing *FLC* and *FRI* (Fig. 2c,d). Thus the autonomous promotion pathway can be blocked by recessive mutations in pathway genes (*ld, fca*, etc.) or dominant genes that have evolved to attenuate this pathway (*FRI*) (details of the interactions of these pathways are discussed in the following section and in Fig. 3). Either type of block to the autonomous pathway can be overcome by vernalization.

Because vernalization-responsive late flowering can be conferred in Arabidopsis either by dominant alleles of *FRI* and *FLC* or by recessive autonomous-pathway mutations, the segregation of the winter-annual habit in crosses between various Arabidopsis lines can mimic the segregation of biennialism in other species. In an F_2 population generated by crossing a *FRI*-containing winter-annual strain with an early flowering laboratory strain lacking *FRI*, the winter-annual trait segregates as a single dominant locus, similar to the behaviour seen in crosses of annual and biennial *H. niger* (Burn *et al*. 1993b; Lee *et al*. 1993; Clarke & Dean 1994). In a population lacking *FRI* and segregating for an autonomous-pathway mutation such as *fca* or *ld*, the winter-annual trait segregates as a single recessive locus, similar to the situation in beet. Biennial strains of diploid wheat and barley contain both a dominant *Vrn-2* allele and recessive *vrn-1* alleles (*Vrn-2/–; vrn-1/vrn-1*) which together confer the winter growth habit. The recessive allele of *vrn-2* which promotes the spring growth habit is epistatic to *vrn-1* and the dominant *Vrn-1* allele which confers the spring growth habit is epistatic to the dominant *Vrn-2* allele (Tranquilli & Dubcovsky, 2000). (Thus plants which are genotypically *Vrn-2/–; Vrn-1/– and vrn-2/vrn-2*; *vrn-1/vrn-1* grow as spring annuals.) These genetic interactions are mimicked in Arabidopsis by *FLC* and autonomous-pathway mutations (these interactions are discussed further below). *Vrn-2* like dominant alleles of *FLC*, and *vrn-1* like recessive autonomous-pathway mutations promote the winter growth habit. Epistatic relationships are also similar to that seen for *Vrn-2* and *Vrn-1*; recessive alleles of *flc* are epistatic to autonomous-pathway mutations and dominant (wild-type) alleles of autonomous-pathway genes are epistatic to dominant *FLC* alleles.

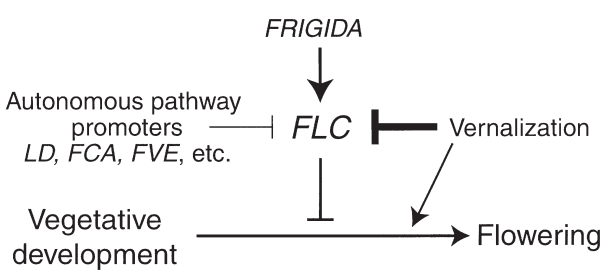

Figure 3. Local model for the regulation of flowering time by *FLC*, *FRI*, the autonomous pathway and vernalization. Epistasis relationships are indicated by line thickness: *FRI* upregulates *FLC* despite the inhibitory effect of the autonomous pathway and vernalization downregulates *FLC* in the presence of *FRI* or mutations in the autonomous pathway.

MOLECULAR GENETICS OF VERNALIZATION

One approach to study the molecular mechanism of vernalization is to identify the gene products involved. Extensive mutant screens have been performed in Arabidopsis to identify flowering-time genes, but such screens are unlikely to reveal vernalization mutants because they have been performed on early flowering strains that have little response to vernalization. Only recently have screens been designed to identify mutations in the vernalization pathway. Chandler, Wilson & Dean (1996) identified several mutants with reduced sensitivity to vernalization by mutagenizing a line containing the *fca* mutation, which confers a late-flowering, vernalization-responsive phenotype, and screening the M_2 generation for plants that remained late flowering after cold treatment. These mutants still show some response to vernalization. Further screens may reveal additional mutations that create completely vernalization-insensitive plants, although it is possible that the vernalization pathway contains genes with overlapping function, and double or higher order mutants will have to be constructed to create a truly vernalization-insensitive plant.

Although genes involved directly in the vernalization pathway have yet to be molecularly identified, significant progress has been made in characterizing the genes that create the vernalization requirement in Arabidopsis. As discussed above, *FRI* and *FLC* interact to create the winter-annual habit in Arabidopsis and both genes have recently been cloned. The *FLC* gene encodes a MADS-domain-containing transcription factor (Michaels & Amasino 1999a; Sheldon *et al*. 1999) and *FRI* encodes a protein without significant identity to proteins of known function (Johanson *et al*. 2000). As discussed below, expression studies of *FLC* and the characterization of null mutants have provided the framework for how *FRI*, autonomous-pathway genes, and vernalization interact to control flowering time (Fig. 3).

Dominant *FLC* alleles, such as those from late-flowering winter-annual ecotypes, encode an active protein that acts to inhibit flowering (Koornneef *et al*. 1994; Lee *et al*. 1994; Michaels & Amasino 1999a). In the absence of *FRI* [early flowering, wild-type laboratory strains such as Columbia are *fri* null mutants (Johanson *et al*. 2000)], *FLC* expression is not detected (Michaels & Amasino 1999a; Sheldon *et al*. 1999). In the presence of *FRI* or an autonomous-pathway mutation, such as *ld* (two situations that cause vernalization-responsive late flowering), *FLC* expression is upregu-

lated. If *FRI*-containing or *ld*-mutant plants are cold treated, however, *FLC* expression is suppressed to undetectable levels. We have also determined that the late-flowering phenotype of *FRI* and certain autonomous-pathway mutations, such as *ld, fca,* and *fve* are eliminated in the presence of a null allele of *flc* (S. C. Michaels and R. M. Amasino, unpublished results). Thus these genes seem to act entirely by altering *FLC* levels. Therefore, eliminating *FLC* expression by cold treatment or by a null mutation is equally effective in eliminating the late-flowering phenotype of *FRI* and certain autonomous-pathway mutations. Furthermore, the late-flowering phenotype of transgenic plants constitutively expressing *FLC* from a heterologous promoter is not suppressed by vernalization (Michaels & Amasino 1999a; Sheldon *et al*. 1999). Thus, the effect of vernalization is due, at least in part, to the suppression of *FLC* expression (Fig. 3). The repression of *FLC* by vernalization has also been observed in biennial Brassicas (Kole *et al*. 2000), providing evidence that *FLC* is a target of the vernalization pathway in cold-responsive species other than Arabidopsis. In fact, the difference in flowering behaviour between winter-annual Arabidopsis and biennial Brassicas may partly be the result of differences in FLC allele strength or regulation of *FLC*. We have transformed a winter-annual (*FRI*-containing) Arabidopsis strain with additional genomic copies of *FLC*. Like the non-transgenic parent, these lines are early flowering after cold treatment; however, the plants with extra copies of *FLC* do not flower in the absence of cold treatment (Fig. 4). Thus, by increasing *FLC* copy number it is possible to convert Arabidopsis from a winter annual to a true biennial (Fig. 5).

These results show that *FLC* is a downstream target of the vernalization pathway and that *FLC* downregulation is necessary for vernalization to be effective. Recent experiments, however, show that the downregulation of *FLC* does not completely account for the vernalization response in Arabidopsis (S. D. Michaels and R. M. Amasino, unpublished). Plants containing a *flc*-null mutation were grown under short days with and without prior cold treatment. (Because Arabidopsis is a facultative long-day plant, flowering occurs much later in short days, making any promotion of flowering by vernalization easier to observe.) After 60 days of cold exposure, *flc*-null mutants show a clear vernalization response in short days; cold-treated *flc*-null plants flowered with fewer than half the number of leaves as non-cold-treated plants. Thus downregulation of *FLC* does not totally account for the vernalization response.

There are two points we wish to emphasize. One is that downregulation of *FLC* in response to cold takes precedence over other aspects of regulation because the increase in *FLC* due to *FRI* or autonomous-pathway mutations is eliminated after vernalization. The other is that the suppression of *FLC* by vernalization is independent of *FRI* and autonomous-pathway genes. A *fri* null plant that also has

Figure 4. Biennial Arabidopsis. *FRI*-containing plants transformed with additional genomic copies of *FLC* do not flower in the absence of cold treatment. In this non-cold-treated plant, the primary meristem senesced after forming more than 100 leaves, the primary leaves have senesced and axillary meristems are forming secondary rosettes.

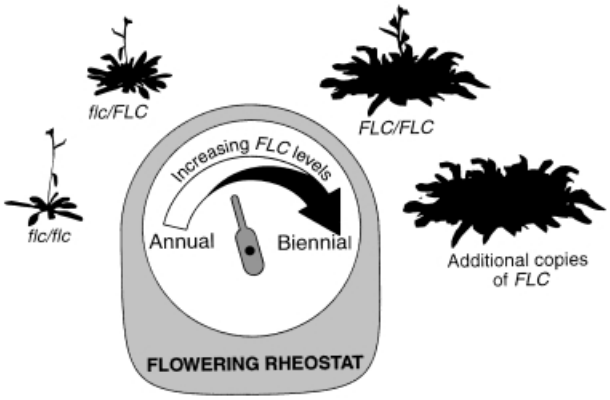

Figure 5. *FLC* delays flowering in a rheostat-like manner. The delay in time to flowering in a *FRI*-containing background is proportional to *FLC* copy number. Transgenic plants containing additional genomic copies of *FLC* do not flower in the absence of cold treatment and thus behave as true biennials (see also Figure 4).

an autonomous-pathway null mutation is late flowering without vernalization and *FLC* is expressed until downregulated by vernalization. In such a line, vernalization cannot be acting through *FRI* or the autonomous-pathway gene that is lacking. Therefore, vernalization appears to be a distinct pathway.

Consistent with the observation that the vernalized state is stable throughout subsequent mitotic divisions, *FLC* levels remain undetectable for the remainder of the plant's life cycle after cold treatment (Michaels & Amasino 1999a; Sheldon *et al*. 1999). The spatial expression pattern of *FLC* is also consistent with it being a target of the vernalization pathway. We have determined the expression pattern of *FLC* using RNA-blot analysis and reporter constructs, and found that strong *FLC* expression is localized to the shoot and root tips (Fig. 6). These are the same tissues that Wellensiek (1964) found were able to respond to vernalization in *Lunaria* .

Of the responses that plants have evolved to environmental stimuli, vernalization remains one of the least understood. Nothing is known of the molecular mechanism of cold perception in vernalization or the signal-transduction pathway that produces the mitotically stable memory of cold exposure. It is now clear, however, that *FLC* plays a central role in creating the vernalization requirement in naturally occurring winter-annual ecotypes of Arabidopsis and late-flowering genotypes containing mutations in autonomous-pathway genes. In these genotypes, *FLC* levels are high and flowering is delayed unless *FLC* expression is suppressed by vernalization. As discussed above, the degree of lateness of non-cold-treated plants is proportional to *FLC* copy number and presumably gene expression levels (Fig. 5) and in fact an increase in *FLC* copy number can convert Arabidopsis from a winter annual into a biennial (Fig. 4)! Regardless of the *FLC* copy number and the consequent extent of a flowering delay, vernalization can fully suppress the late-flowering effects of *FLC*. This vernalization suppression coincides with the disappearance of *FLC* transcripts. A model of vernalization consistent with these observations is that there is a cold-perception and cold-signal-transduction pathway that culminates in an epigenetic switching off of *FLC* expression (and possibly the switching on and off of other genes as well) which in turn renders the meristem competent to initiate flowering. Unravelling the molecular details of this pathway will hopefully reveal how plants remember winter.

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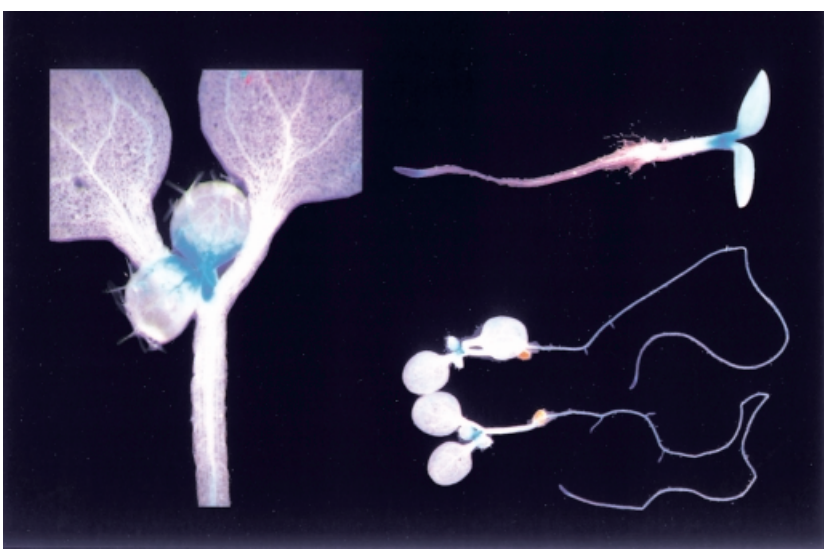

Figure 6. Localization of *FLC* by GUS staining.

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