

# DNA methylation, vernalization, and the initiation of flowering

(thermoinduction/5-azacytidine/*Arabidopsis thaliana*)

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**ABSTRACT** Late-flowering ecotypes and mutants of *Arabidopsis thaliana* and the related crucifer *Thlaspi arvense* flower early after cold treatment (vernalization). Treatment with the DNA demethylating agent 5-azacytidine induced nonvernalized plants to flower significantly earlier than untreated controls. Cytidine at similar concentrations had no effect on time to flower. In contrast, late-flowering mutants that are insensitive to vernalization did not respond to 5-azacytidine treatment. Normal flowering time was reset in the progeny of plants induced to flower early with 5-azacytidine, paralleling the lack of inheritance of the vernalized condition. *Arabidopsis* plants, either cold-treated or 5-azacytidine-treated, had reduced levels of 5-methylcytosine in their DNA compared to nonvernalized plants. A *Nicotiana plumbaginifolia* cell line also showed a marked decrease in the level of 5-methylcytosine after treatment with either 5-azacytidine or low temperature. We suggest that DNA methylation provides a developmental control preventing early flowering in *Arabidopsis* and *Thlaspi* ecotypes. Vernalization, through its general demethylating effect, releases the block to flowering initiation. We propose that demethylation of a gene critical for flowering permits its transcription. We further suggest, on the basis of *Thlaspi* data, that the control affects transcription of kaurenoic acid hydroxylase, a key enzyme in the gibberellic acid biosynthetic pathway.

In many plants the transition of the shoot apical meristem from the vegetative to the flowering state occurs in response to environmental cues. Day length is one such cue, some plants requiring short, others long, days to initiate flowering. The site of perception of day length is the leaf, where a signal is generated and subsequently translocated to the shoot apex, where a new pattern of cell division forms the flowering meristem that subsequently produces the inflorescence (1).

Exposure to low temperature can induce flowering in many temperate monocots and dicots, the cold treatment being termed vernalization (1). Experiments involving cooling treatments localized to parts of plants have shown that the shoot apical meristem is itself a site of perception of the low-temperature treatment (2, 3). Wellensiek (4) provided evidence that cell division during vernalization is necessary for thermoinduction in *Lunaria annua* and that flowering structures are ultimately derived from the mitotically active cells that were subjected to vernalizing temperatures. He concluded that thermoinduction is a cell-autonomous process that is mitotically propagated.

The observation that tissues other than the shoot apical meristem also have the capacity to support thermoinductive responses to vernalization is consistent with the concept of mitotic transmission. Shoots regenerated from leaf cuttings of both *L. annua* (4) and *Thlaspi arvense* (3) exhibited a developmental state identical to that of plants from which they were obtained: flowering shoots developed from leaves of cold-treated plants, whereas only vegetative rosettes de-

veloped from the leaves of nonvernalized plants. These data demonstrate a mitotic memory of thermoinduction that is stable through many cell divisions and even through changes in the state of cellular differentiation.

In all plants that require vernalization to flower, the pattern of development is reset to vegetative growth in the progeny of the thermoinduced plant; progeny plants must also be vernalized in order to flower (1). Thermoinduction of flowering is an epigenetic process, restricted to a single sexual generation.

Although mechanisms for the epigenetic control of gene expression are not well understood, there is increasing evidence that the pattern of DNA methylation is important (5). In plants and animals, the cytosine residue of a CG dinucleotide can be methylated, and in plants methylation of CNG motifs also occurs (6). The pattern of methylation is propagated by the activity of a methyltransferase enzyme that prefers a hemimethylated double-stranded sequence as substrate (7). Methylation pattern is maintained with high fidelity through each DNA replication cycle and is transmitted to both daughter cells resulting from a mitotic cell division (5).

The lack of transcriptional activity has been associated with methylation of cytosine residues within the promoter region of a gene (8, 9). DNA-binding proteins, including transcription factors, can be sensitive to the presence of methylated cytosines in DNA (10), and this may be what prevents transcription.

Treatment of plant and animal cells with 5-azacytidine (5-azaC) results in the demethylation of DNA directly by incorporation of the analogue in place of cytosine during DNA replication (11, 12) and indirectly by inhibition of the action of the methyltransferase enzyme (13). Demethylation of DNA by 5-azaC has been correlated with induction of transcription in a number of gene systems in plants and animals (14, 15).

We show that low-temperature treatment results in extensive demethylation of DNA and we propose that flowering promotion is caused by demethylation of the promoter of a gene or genes critical for the induction of flowering. We have support for this hypothesis from experiments in which 5-azaC treatment substituted for vernalization in accelerating flowering.

## MATERIALS AND METHODS

**Plant Material.** The late-flowering ecotypes Pitztal (Pi) and Kiruna-2 (Kr-2) originated from L. Laibach (16). Late-flowering mutants *fca*, *fy*, *ft*, *fd*, and *gi* (*fb*) of the early-flowering ecotype Landsberg erecta (Ler) (17) were kindly provided by Maarten Koornneef and *fca-7* was provided by David Smyth. *T. arvense* L. was the CR<sub>1</sub> inbred line (18).

Abbreviations: 5-azaC, 5-azacytidine; Pi, Pitztal; Kr-2, Kiruna-2; Ler, Landsberg erecta; GA, gibberellic acid; BT, time to bolt; LN, leaf number at flowering.

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*Arabidopsis* was grown in test tubes in artificially lit growth cabinets under long days (16 hr light, 8 hr dark at 23°C) (19, 20) with fluorescent lights at a photosynthetic photon flux density of 200  $\mu\text{M}/\text{m}^2$  per s. Germinating seedlings were vernalized in the dark at 4°C for 20–30 days. The number of days after plants were removed from the cold treatment until stem elongation (bolting) began was used as a measure of flowering time (BT). Leaf number (LN) was the number of rosette leaves at the time of flowering.

**Regeneration of Shoots from *Arabidopsis* Roots.** Excised roots were placed on a callus-inducing medium for 4 days at 23°C and then transferred to a shoot-inducing medium. After 18 days, regenerated shoots were excised and placed in 9-cm Petri dishes containing growth medium (21). Each Petri dish contained 10 shoot explants and all treatments had three replicates.

**5-azaC Treatments.** Sterile *Arabidopsis* seeds were imbibed at 23°C on filter paper soaked with fresh 5-azaC solution (50  $\mu\text{M}$ ). The seeds were transferred daily to new filter paper containing fresh 5-azaC solution. After 5 days the germinated seeds were transferred to growth medium.

*Thlaspi* seeds were transferred daily to filter paper saturated with fresh 5-azaC solution. After 5 days the seedlings were transplanted to pots containing vermiculite.

**Effect of 5-azaC and Vernalization on 5-Methylcytosine Levels.** Cultures (*Nicotiana plumbaginifolia* line NpT<sub>5</sub>) (22) were grown in liquid CS5 medium and were maintained at 24°C with constant shaking for 7 days. For the 5-azaC treatment, cells were grown under similar conditions except that 5-azaC solution was added 3 days after subculture and cells were harvested 4 or 9 days after treatment. For the cold treatment, the cell suspension cultures were transferred to a cold room at 6°C for 10 days.

Harvested cells were frozen at -80°C and ground in liquid nitrogen, and DNA was extracted (23). The crude DNA pellet was treated with 0.5 M NaOH at 37°C for 1 hr to remove RNA and DNA precipitated with ethanol; 20–60  $\mu\text{g}$  of DNA was hydrolyzed with 12 M perchloric acid (24).

The residue was resuspended in 100  $\mu\text{l}$  of H<sub>2</sub>O and analyzed for the relative amounts of cytosine and 5-methylcytosine (280 nm) using HPLC (24) with a strong cation-exchange column maintained at 60°C. The detector responses for peaks with the same retention times as cytosine and 5-methylcytosine standards were integrated and the percentage of cytosines that was methylated was calculated. Each treatment had two replicates and each sample was analyzed twice.

## RESULTS

**5-azaC Promotes Flowering in Vernalization-Requiring Late-Flowering Ecotypes and Mutants of *Arabidopsis*.** The late-flowering *Arabidopsis* ecotype Pi responds to a vernalization treatment (21 days at 4°C) such that time for 50% of plants to bolt was reduced from 110.6  $\pm$  11.7 (mean  $\pm$  SEM) days to 20  $\pm$  0.57 days after 21 days at 4°C. Initiation of flowering of Pi was also accelerated by treatment with 5-azaC. Imbibition of germinating Pi seed in the presence of 50  $\mu\text{M}$  5-azaC reduced time to bolt to 71.1  $\pm$  2.67 days for the first 50% of plants. Other concentrations of 5-azaC (25  $\mu\text{M}$  to 5 mM) were less effective, and cytidine over a similar range of concentrations did not promote flowering. Although the 5-azaC treatment caused a large decrease in time of flowering, the reduction was not as great as the 21-day vernalization treatment.

Kr-2, another vernalization-responsive late-flowering ecotype, also flowered early after exposure to 5-azaC. 5-azaC treatment or vernalization (4°C for 21 days) reduced flowering times from 36.4  $\pm$  1.3 days (nonvernalized controls) to 30.1  $\pm$  0.8 and 21.5  $\pm$  1.5 days, respectively.

Late-flowering mutants of the *Arabidopsis* ecotype Ler have differing responses to vernalization (20, 25, 26); *fca* and *fy* flower much earlier following vernalization, whereas *fd*, *ft*, and *gi* (*fb*) are either nonresponsive or only slightly responsive (similar to the Ler control). Treatment of germinating seeds with 5-azaC led to markedly earlier floral initiation and a reduction in the number of rosette leaves formed at flowering in those mutants that were responsive to vernalization (Table 1). In all mutants, except *ft*, the leaf number at flowering was correlated with bolting time following either vernalization or 5-azaC application. The mutant *ft* showed no significant difference in the number of leaves formed in response to either vernalization or 5-azaC treatment. The time of *ft* to bolt, however, was significantly reduced following either treatment, suggesting that *ft* may be a different class of mutant, perhaps one with an altered period of juvenility.

**Root Meristems Respond to Cold Treatment.** Pi plants were vernalized during germination and roots excised 3 weeks later were cultured to regenerate shoots. All 30 shoots flowered within 2 weeks following transfer to growth medium, whereas none of the controls (derived from the roots of germinating seed not exposed to cold treatment) flowered even after 5 weeks. These data show that the cold-treated root meristem in the germinating seed gave rise to cells that perpetuated an

Table 1. Treatment of Ler late-flowering mutants with 5-azaC or vernalization (Vern.)

Genotype	Parameter	Control	5-azaC	% reduction	
				5-azaC	Vern.
Ler	BT	22.1 $\pm$ 0.2	21.6 $\pm$ 0.2	2.3	12.1
	LN	9.1 $\pm$ 0.1	8.5 $\pm$ 0.4	6.6	6.0
<i>fca-1</i>	BT	33.2 $\pm$ 0.7	25.8 $\pm$ 0.4*	22.3	33.0
	LN	15.9 $\pm$ 0.6	11.3 $\pm$ 0.4*	28.9	42.7
<i>fca-7</i>	BT	41.6 $\pm$ 1.8	28.9 $\pm$ 1.1*	30.5	52.4
	LN	18.3 $\pm$ 0.9	12.6 $\pm$ 0.5*	31.1	52.7
<i>fy</i>	BT	32.2 $\pm$ 0.7	28.9 $\pm$ 1.1*	10.2	33.4
	LN	14.3 $\pm$ 0.3	10.6 $\pm$ 0.5*	25.8	32.7
<i>ft</i>	BT	40.9 $\pm$ 0.7	45.2 $\pm$ 1.2*	-10.5	19.3
	LN	17.8 $\pm$ 0.6	17.4 $\pm$ 0.3	2.2	6.8
<i>fd</i>	BT	28.4 $\pm$ 0.3	33.2 $\pm$ 0.9*	-16.9	12.7
	LN	13.4 $\pm$ 0.4	15.5 $\pm$ 0.6*	-15.7	10.9
<i>fb</i>	BT	31.6 $\pm$ 0.8	29.9 $\pm$ 1.2	5.4	7.5
	LN	14.6 $\pm$ 0.5	14.2 $\pm$ 0.5	2.7	6.4

Seeds of the late-flowering mutants were imbibed for 4 days in either distilled water (control) or 50  $\mu\text{M}$  5-azaC. These were transferred to test tubes containing solid growth medium grown under fluorescent lights. A vernalized control (24 days at 4°C) was also included. Data are expressed as mean  $\pm$  SEM. Values within rows followed by \* are significantly different at the  $P = 0.05$  level as determined by the Student–Newman–Keuls multiple-range test.

altered state and that shoot meristems organized from these cells produced plants that flowered without vernalization. We found that excised roots that had not been cold treated could produce shoots with early flowering if a cold treatment of 21 days was applied shortly after the initiation of culture (provided cell divisions were actively occurring).

**5-azaC Also Promotes Early Flowering in Root-Derived Shoots.** When shoots were regenerated from roots of 3-week-old Pi plants that had been treated with 50  $\mu$ M 5-azaC during germination, a significant increase in flowering was observed; 25%  $\pm$  5% of the treated plants had flowered after 7 weeks, whereas none of the control plants had flowered by this time. A similar effect was observed when excised roots from 3-week-old Pi or *fca-7* plants were placed on callus-inducing medium containing different concentrations of 5-azaC for 4 days prior to the regeneration of shoots (Table 2). Significant promotion of flowering over a broad range of concentrations was observed for both lines. These experiments involving the formation of a secondary meristem show that cells in any cell lineage leading to an inflorescence can respond to either vernalization or 5-azaC treatment.

**The Progeny of Plants Induced to Flower with 5-azaC Are Not Early Flowering.** The promotive effects of vernalization on flowering are not carried into subsequent generations; plants of each generation need to be vernalized for early flowering to occur (1). Progeny from plants of the Pi ecotype and the mutant *fca-7*, induced to flower early with 5-azaC treatment, flowered at the same time as nonvernalized controls (Table 3), showing that the promotion of flowering by 5-azaC is an epigenetic effect, as is vernalization.

**5-azaC Treatment Substitutes for Vernalization in *T. arvense*.** *T. arvense*, a species in the same family as *Arabidopsis*, shows a strong vernalization response. At 21°C the shoot apex does not change from vegetative to reproductive development until 170 days after planting. Vernalization for 4 weeks reduces flowering time to ca. 90 days. 5-azaC treatment of germinating *Thlaspi* seeds also advances initiation of flowering; all plants were bolting, and 62% of plants had open flowers 91 days after 5-azaC treatment (Table 4, Fig. 1).

The extent of advancement of flowering time is dependent on 5-azaC concentration, 250  $\mu$ M being optimum. Higher concentrations of 5-azaC were inhibitory to bolting and flower development probably because of nonspecific toxic effects of 5-azaC. Cytidine over a similar range of concentrations had no effect on time to flower of *Thlaspi*.

**5-azaC and Vernalization Result in Demethylation of DNA.** Treatment of animal and plant cells with 5-azaC results in a reduction in the proportion of methylated cytosine in DNA (11, 12). If vernalization and 5-azaC promote flowering through similar mechanisms, then vernalization might also be expected to reduce DNA methylation. Cytosine methylation was measured indirectly with a methylation-sensitive restriction enzyme or directly using HPLC assay. The restriction enzymes *Msp* I and *Hpa* II were used to monitor the meth-

Table 2. Effect of 5-azaC treatment of excised roots of Pi and *fca-7* on subsequent flowering in regenerated shoots

5-azaC, $\mu$ M	% flowering	
	<i>fca-7</i>	Pi
0	17 $\pm$ 13	0 $\pm$ 0
50	60 $\pm$ 0	40 $\pm$ 7
100	50 $\pm$ 10	28 $\pm$ 2
250	67 $\pm$ 13	20 $\pm$ 2
500	37 $\pm$ 3	0 $\pm$ 0

Roots of plants were excised and placed on a callus-inducing medium containing various concentrations of 5-azaC for 4 days. The number of shoot explants that produced flower buds was assessed 4 and 7 weeks after transfer to shoot-inducing medium for *fca-7* and Pi, respectively. Data are expressed as mean  $\pm$  SEM.

Table 3. Time to flower of the progeny of 5-azaC-treated Pi and *fca-7* plants

Treatment	BT, days	LN
Pi		
Progeny of a		
5-azaC-treated plant	59.9 $\pm$ 2.6	—
Nonvernalized control	67.2 $\pm$ 4.8	—
Vernalized control	17.1 $\pm$ 0.6*	—
<i>fca-7</i>		
Progeny of a		
5-azaC-treated plant		
i	45.6 $\pm$ 2.0	19.4 $\pm$ 0.7
ii	50.5 $\pm$ 2.6	18.2 $\pm$ 0.8
iii	49.1 $\pm$ 2.9	18.2 $\pm$ 1.0
Nonvernalized control	47.6 $\pm$ 1.1	18.1 $\pm$ 0.8
Vernalized control	26.0 $\pm$ 1.1*	10.8 $\pm$ 0.6*

Twenty seeds from one early-flowering 5-azaC-treated Pi plant and three *fca-7* plants were grown in test tubes under either fluorescent light supplemented with incandescent light (Pi) or fluorescent lights alone (*fca-7*). A nonvernalized and a vernalized control (24 days at 4°C) were also included. Data are expressed as mean  $\pm$  SEM. Values within a column followed by \* are significantly different at the  $P = 0.05$  level as determined by the Student–Newman–Keuls multiple-range test.

ylation status of CCGG sites within the centromeric satellite repeats of *Arabidopsis* DNA. *Hpa* II will cut DNA only if all cytosines in the recognition site are unmethylated, whereas *Msp* I cleaves whether the inner C is methylated or not.

Increased digestion with *Hpa* II of DNA from *fca-7* plants, following vernalization or treatment with 5-azaC, indicated a reduction in methylation of the satellite repeat region in response to both treatments (Fig. 2). The 5-azaC treatment caused more demethylation since the extent of digestion by *Hpa* II was greater in DNA extracted following vernalization.

In *Arabidopsis* DNA, only 6% of cytosines are methylated (30) compared to 30–35% in *Nicotiana* (14). The low level in *Arabidopsis* makes it difficult to measure small changes in the percentage of methylated cytosine. We therefore used *N. plumbaginifolia* suspension culture cells to investigate whether low temperature affected the DNA methylation status of the cells. Low temperature and 5-azaC substantially reduced the proportion of methylated cytosine in DNA (Table 5). The effect of 5-azaC was greater than the cold treatment; 4- or 9-day 5-azaC treatment resulted in a 37% or 55% reduction in methylated cytosine, respectively, whereas low temperature reduced the level by 22%.

These results demonstrate that low temperature and 5-azaC treatment decrease the level of DNA methylation in germinating seedlings and cell culture and that low-temperature demethylation is not restricted to vernalization-

Table 4. Effect of 5-azaC on reproductive development in *Thlaspi*

Parameter	5-azaC, $\mu$ M				
	0	50	100	250	500
% bolting	0	92.3	95.1	100.0	83.7
Median stem height	0 <sup>a</sup>	58.0 <sup>b</sup>	91.0 <sup>bc</sup>	155.0 <sup>c</sup>	28.0 <sup>d</sup>
% flower buds	0	15.4	31.7	33.3	51.2
% open flowers	0	34.6	43.9	61.9	0
% siliques	0	19.2	21.6	26.2	0

Plants were grown at 21°C and observations were made 13 weeks after planting. Unvernalized *Thlaspi* takes 24 weeks to flower under these conditions. Values followed by different letters are significantly different at the  $P = 0.05$  level as determined by the method of Gibbons (27) following analysis of variance using the Kruskal–Wallis test (nonparametric test using ranked sums).



FIG. 1. Response of *T. arvense* to treatment with 250  $\mu$ M 5-azaC. NV refers to plants that were neither vernalized nor 5-azaC treated. Plants are 13 weeks after planting.

sensitive species or to those cells that respond to vernalization by advancing flowering.

### DISCUSSION

Our experiments, using two different plant species in which low temperature induces flowering, have shown that 5-azaC treatment partially substitutes for cold treatment in the promotion of flowering. We have shown that 5-azaC treatment parallels vernalization in a number of key properties. Both treatments are meristem specific; they are cell division dependent and cell lineage propagated, and their effects are not inherited through successive sexual generations.

Since 5-azaC incorporation into DNA results in demethylation, the inference can be drawn that the methylation status of particular DNA sequences controls the potential for transition from vegetative to reproductive development. Our presumption is that methylated nucleotides in the promoter region of a flowering initiation gene(s) prevents transcription.

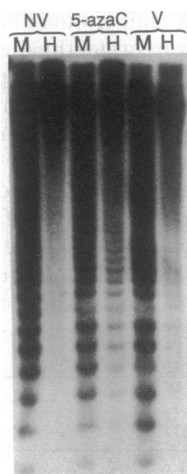


FIG. 2. Total genomic DNA was isolated from *Arabidopsis* mutant *fca-7* that either had been grown at 23°C for 21 days (nonvernalized) or had been exposed to low temperature (21 days at 4°C) or 5-azaC (4 days) before being transferred to 23°C for 21 days. DNA was digested with *Msp* I (M) or *Hpa* II (H), 5–10  $\mu$ g of digested DNA was run on an 0.8% gel overnight (30–40 V), and blotted (28) onto nylon membranes. A 2.5-kilobase fragment containing a single 180-base-pair satellite repeat from *Arabidopsis* (kindly supplied by Eric Richards) was labeled with [<sup>32</sup>P]dCTP using the random primer method (29) and used as a probe. V, vernalized; NV, nonvernalized.

We further postulate that this methylation block is removed by either low temperature or 5-azaC treatment. Since methylation patterns are transmitted through mitotic divisions, with members of a cell lineage having the same pattern of methylation, we relate the initial demethylation event to the subsequent meristem transition.

Our findings of the inheritance of the thermoinduced state through extensive numbers of cell divisions, and even through dedifferentiation and subsequent redifferentiation of cells (Table 2), fit well with the hypothesis that a change in the DNA methylation status mediates thermoinduced flower initiation. This explains why thermoinduction does not appear to be graft transmissible (4), although Lang (ref. 1, p. 1492) does cite an experiment of Melchers that he concludes argues for a transmissible “vernalinalin.” Information conserved in a DNA methylation pattern is cell autonomous and not mobile between cells. Vernalization treatment must be applied to the progenitor cells of the inflorescence cell lineage, whether they occur in shoot apical meristems or in secondary meristems derived from tissue generated from either root or shoot meristems. Cells in the root meristem switch to the thermoinduced state in response to treatment, but their flowering potential can only be expressed following appropriate morphogenesis.

Our hypothesis explains the temporal disjunction of a vernalization treatment and the much later onset of flowering in the mature stage of development of the plant. This disjunction has been difficult to rationalize in terms of specific cell products or generalized hormone effects.

Table 5. Percent 5-methylcytosine in the DNA of *Nicotiana* cells exposed to 100  $\mu$ M 5-azaC or low temperature

Treatment	% 5-methylcytosine as a proportion of total cytosine		
	Exp. 1	Exp. 2	Exp. 3
Control	35.0	34.1	33.5
5-azaC			
4 days	21.6		
9 days		15.3	15.4
Cold	27.5	27.4	25.3

DNA ( $\approx$ 50  $\mu$ g) was hydrolyzed with 12 M perchloric acid and the relative amounts of cytosine and 5-methylcytosine were determined by HPLC. Each value represents the mean of two replicate injections.

The reason that the effectiveness of vernalization increases with the length of the cold treatment may be related to the number of cell division cycles needed to dilute out the initial methylated DNA strands in the dividing aggregate of cells in the inflorescence lineage. The multiplicity of cells and their probability of entry into cell division may also explain the partial penetrance of the 5-azaC treatment.

The maintenance methylases in *Arabidopsis* and *Thlaspi* may be cold sensitive and thus methylation could be uncoupled from DNA replication by the cold treatment resulting in the newly synthesized strands being unmethylated. Cold sensitivity of methyltransferase may be a general property of this enzyme in plants, since low-temperature treatment of *N. plumbaginifolia* cells, a species not responsive to vernalization, also results in a reduction in DNA methylation. Temperature-induced demethylation presumably affects genome sequences in general and may alter the regulatory regions of a number of genes. The fact that higher concentrations of 5-azaC are toxic may reflect a perturbation of the normal pattern of gene expression, although at concentrations of 5-azaC that resulted in a decreased time to flower, no other major effects of the drug were observed.

The fact that the effects of vernalization and 5-azaC are epigenetic, treatments needing to be provided in each sexual generation, further supports the methylation control hypothesis since methylation patterns are reset every generation (31). Control of flowering initiation by the methylation status of the DNA sequence in the promoter(s) of key genes could apply to all species of the wide range of monocots and dicots that respond to vernalization. An experiment by other workers in this laboratory with two vernalization-sensitive wheat cultivars showed that 5-azaC partially substituted for vernalization. However, subsequent experiments with the same genotypes have failed to confirm this response (R. D. Brock and J. L. Davidson, personal communication), the reasons being as yet not understood.

**Biochemical Basis to the Model.** In many plants, including *Arabidopsis* and *Thlaspi*, the vernalization requirement can be bypassed by application of gibberellic acid (GA), resulting in flowering initiation at a time similar to that of vernalized plants (1, 20, 32). Also in *Thlaspi*, kaurenoic acid metabolism, a step in the GA biosynthetic pathway, is under thermoregulation in the apex but not in the leaves (33). The enzyme converting kaurenoic acid to 7 $\beta$ -OH kaurenoic acid is induced by low temperature (34). We have preliminary evidence that the same step in GA biosynthesis is under thermoinductive regulation in *Arabidopsis*. In *Thlaspi* and *Arabidopsis* the promoter of the meristem kaurenoic acid hydroxylase gene may be inactivated by methylation. The implication is that until these methyl groups are removed, by vernalization or by 5-azaC, the gene is not transcribed.

Mutants at different loci, such as *fca*, *fy*, and *fve*, all have similar responses to vernalization. Furthermore, all recovered alleles of each of these loci are vernalization sensitive (26). This suggests that *Arabidopsis* plants may have an underlying vernalization block (25, 26) but that in early-flowering ecotypes, such as Ler, there are metabolic pathways circumventing the specific block (Fig. 3). The late-flowering vernalization-sensitive lines may have mutations blocking the shunt

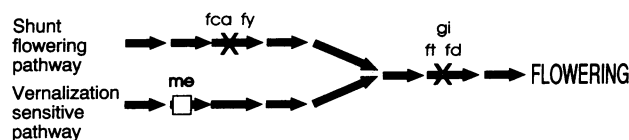


FIG. 3. Pathways to flowering in Ler. Individual mutations giving rise to late flowering are indicated by X and the methylation block in the promoter of a gene in the vernalization pathway is shown as a square (me). Mutant stocks are described by Koornneef *et al.* (26).

pathway. Landsberg grown under low light intensities or short days does show a low-level vernalization response. Other late-flowering loci—e.g., *ft*, *fg*, *fd*—that have a small vernalization response, equivalent to Landsberg, may control either later steps in the GA pathway or steps in other pathways.

We conclude that DNA methylation plays a key role in the control of flowering initiation in plants responding to a low-temperature cue. Our proposal that this is mediated through control of a step in an apex-specific GA biosynthetic enzyme gene is open to experimental analysis.

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