

# ***Arabidopsis* mutants showing an altered response to vernalization**

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## **Summary**

**Flowering in many plant species is accelerated by a long period of cold temperature, known as a vernalization period. This research investigates how this cold temperature signal is perceived by plant cells and the mechanism by which it influences the transition to flowering. Mutagenesis of the late-flowering, vernalization-responsive, *Arabidopsis* mutant, *fca*, has yielded five independent mutations (termed *vrn* mutations) conferring an altered vernalization response. Allelism tests showed that these mutations fall into at least three complementation groups defining three loci named *VRN 1*, *2* and *3*. The *vrn1* and *vrn2* mutations did not affect the acclimation response as judged by expression of cold-induced transcripts and freezing tolerance assays. *vrn1-1* affected the short-day vernalization response of Landsberg *erecta* and reduced the vernalization response of other late-flowering *Arabidopsis* mutants. The acceleration of flowering by GA<sub>3</sub> was not affected by *vrn1-1*. The *VRN 1* locus was mapped to chromosome 3.**

## **Introduction**

In many plant species the transition from vegetative to reproductive growth is strongly influenced by environmental conditions, such as cold temperature and day length. However, the molecular mechanisms that regulate this transition are still largely unknown (Bernier, 1988; Evans, 1960; Napp-Zinn, 1987). *Arabidopsis thaliana* provides an excellent system with which to carry out a molecular genetic analysis of the control of the floral transition as the flowering time is affected by environmental conditions and many loci and mutations that influence *Arabidopsis* flowering time have been identified (reviewed in Haughn *et al.*, 1995; Martinez-Zapater *et al.*, 1994). In addition, genes known only by their mutant phenotype can be cloned using a map-based cloning strategy. There are a number

of mutations conferring a late-flowering phenotype, the majority of which have been isolated in the Landsberg *erecta* (*Ler*) ecotype. The late-flowering mutations fall into 12 complementation groups and result in differential responses to cold temperature (vernalization) and day length (Martinez-Zapater and Somerville, 1990; Koornneef *et al.*, 1991). These mutations are being extensively characterized (Chandler and Dean, 1994; Martinez-Zapater *et al.*, 1995) as it is likely they identify genes involved in regulating the timing of the floral transition in response to developmental and environmental (internal and external) signals. The genes corresponding to the late-flowering loci have been or are being cloned (Lee *et al.*, 1994; Putterill *et al.*, 1995) and double mutants are being constructed with some of the many mutations previously isolated in the *Ler* ecotype. This allows interactions between late-flowering mutations and other hormonal or meristem identity genes to be analysed, without complications of modifications to the flowering time phenotype from alleles of modifier loci present in other ecotypes.

A vernalization treatment (2–8 weeks at 4°C) results in accelerated flowering for the majority of the late-flowering mutants, with the *fca* mutant showing the greatest response (Koornneef *et al.*, 1991; Martinez-Zapater and Somerville, 1990). The vernalization response is thought to be perceived by dividing cells of the meristem (Metzger, 1988; Schwabe, 1954) and there is a quantitative relationship between the length of the vernalization period and the acceleration of flowering time (Napp-Zinn, 1987). In addition, the effect of vernalization treatment may be transmitted through mitosis but not through meiosis (Evans, 1960). Recently, Burn *et al.* (1993) have proposed that DNA methylation is involved in the vernalization mechanism.

In order to fully elucidate the mechanism of perception of the cold temperature signal and to understand how this accelerates the transition of the meristem to floral development, we have isolated and characterized *Arabidopsis* mutants having a reduced response to vernalization. We chose to mutagenize the late-flowering *fca* line as *fca* plants exhibit a consistently strong vernalization response and the *fca* mutation is in the well-characterized *Ler* background. Five independent mutants, falling into at least three complementation groups were isolated from 36 000 M<sub>2</sub> seedlings. Here we present a characterization of two of the mutants in terms of their flowering time, vernalization response, and their effect on acclimation, another cold temperature response. The *vrn1* mutation was mapped to chromosome 3. *vrn1* was further characterized to

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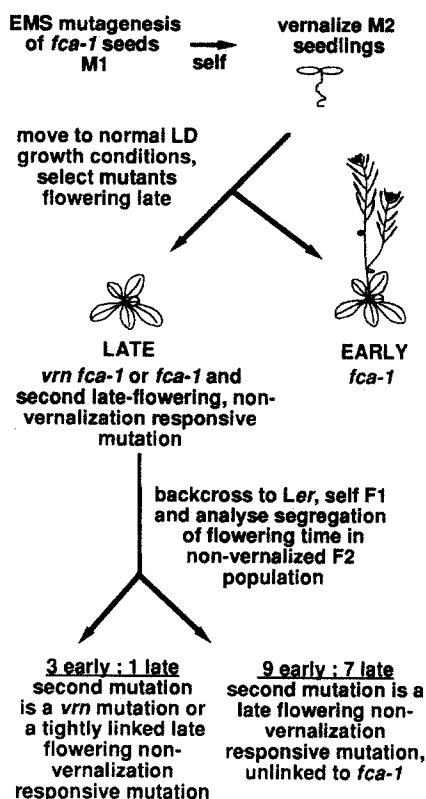
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determine the effect of GA<sub>3</sub> on its flowering time and to study its effect on the vernalization response of other late-flowering mutants.

## Results

### Isolation of mutations that reduce the vernalization response of *fca-1*

To identify mutations in genes needed for perception of or response to vernalization (*vrn* mutations), *fca-1* seed was mutagenized with EMS and 120 vernalized progeny from each of 300 bulked M<sub>2</sub> families (36 000 M<sub>2</sub> plants in total) were grown and individuals flowering later than the vernalized *fca-1* controls identified. Following progeny testing of M<sub>3</sub> seed from 65 such plants, 18 individuals were selected which showed a heritable and significant late-flowering phenotype following vernalization. A mutation that specifically disrupts the vernalization response of *fca* plants should cause vernalized *fca* plants to flower late, but should not cause late-flowering on its own in the *Ler* background. Certain late-flowering mutants, such as *co*, *gi*, etc., flower later than *fca* or *Ler* after vernalization (Koornneef *et al.*, 1991), and *fca* plants containing these mutations would also be isolated from a screen for mutant *fca* plants flowering late after vernalization. It was therefore necessary to determine whether the EMS mutations specifically affected the vernalization response or represented non-vernalization-responsive late-flowering mutations. This was performed by backcrossing the mutants to *Ler* and scoring the F<sub>2</sub> population for flowering time in the absence of a vernalization treatment (illustrated in Figure 1). Depending on the segregation ratios obtained, predictions can be made about the type of mutation identified. A segregation ratio of 3:1 early- to late-flowering plants would indicate that only *fca* was segregating to cause late-flowering without vernalization and that the new mutation caused late-flowering after vernalization by reducing the vernalization response of *fca*. Such mutations were classified as *vrn* mutations. A 3:1 segregation ratio would also result from a second mutation conferring non-vernalization responsive late-flowering that was tightly linked to *fca-1*. However, no mutation causing late-flowering, closely linked to *fca*, has been identified by previous screens. A segregation ratio of 9:7 early to late-flowering would indicate that the new mutation was recessive, also conferred late-flowering, and was unlinked to *fca-1*. Intermediate ratios would indicate the new mutation conferred late-flowering but was linked to varying degrees to *fca-1*. Table 1 shows the segregation for flowering time in the non-vernalized F<sub>2</sub> progeny of the 18 putative *vrn* mutants crossed to *Ler*. F<sub>2</sub> progeny from seven of the crosses segregated early- to late-flowering individuals with a ratio not significantly different from 3:1. Six gave progeny segregating with a ratio not significantly



**Figure 1.** Scheme for isolation of mutations reduced in vernalization response.

Following a vernalization treatment late-flowering mutants were isolated from an *fca-1* M<sub>2</sub> population. The late-flowering individuals were crossed to *Ler* plants and the flowering time of F<sub>2</sub> plants was scored. A ratio of 3:1 early to late-flowering, in the absence of vernalization, is indicative of a line carrying only one late-flowering mutation, namely, *fca-1* and a second mutation specifically affecting the vernalization response. It could also result from the presence of a second late-flowering mutation, tightly linked to *fca*, that does not respond to vernalization.

different from 9:7. Ratios for F<sub>2</sub> populations derived from 47A2,49 and 35D1,19, 30D2,52, 26A2,52 and 50A1,41 were significantly different from 3:1 and 9:7 early to late-flowering. Apart from 47A2,49, only mutants segregating 3:1 when crossed to *Ler* were analysed further.

*vrn* mutations are recessive and represent at least three distinct loci

The *vrn* mutants were backcrossed to *fca-1* and the flowering time in vernalized F<sub>2</sub> progeny scored. The mutants all segregated with ratios not significantly different to 3:1 early to late-flowering (see Table 2) indicating that the mutations are completely recessive.

The putative *vrn*, *fca* mutants were crossed to each other and the flowering time of F<sub>1</sub> plants and F<sub>2</sub> progeny scored. The segregation of flowering time in the F<sub>2</sub> generation is shown in Table 2. All four mutations isolated from the same M<sub>2</sub> family (47A)–47A1,7,7, 47A2,19, 47A2,39 and

**Table 1.** Segregation for flowering time in F<sub>2</sub> progeny from putative *vrn* mutants backcrossed to *Ler*

Mutant	Segregation ratio (early:late)	$\chi^2_{3:1}$	$\chi^2_{9:7}$
47A1,7,7	123:35	$P > 0.05$	-
47A2,19	230:70	$P > 0.05$	-
47A2,39	275:72	$P > 0.05$	-
47A2,49	249:48	$P > 0.05$	-
8A1,20	180:59	$P > 0.05$	-
39A1,1	233:67	$P > 0.05$	-
57D1,33	123:53	$P > 0.05$	-
34C1,23	136:43	$P > 0.05$	-
37C1,9	172:128	-	$P > 0.05$
15B2,25	166:133	-	$P > 0.05$
38A2,45	146:143	-	$P > 0.05$
5D2,12	184:114	-	$P > 0.05$
5D2,17	171:128	-	$P > 0.05$
9A1,46	50:29	-	$P > 0.05$
35D1,19	168:35	$P < 0.02$	-
30D2,52	164:75	$P < 0.05$	-
26A2,52	207:91	$P < 0.05$	-
50A1,41	114:65	-	$P < 0.05$

Plants having leaf numbers greater than those of *Ler* control plants were scored as late flowering. F<sub>2</sub> plants were not vernalized.

47A2,49 were found to be allelic and define the locus *VRN1*. It is likely that they all represent the same mutagenic event. The F<sub>2</sub> progeny of crosses between 8A1,20 and members of the 47A family segregated 9:7 early to late and 8A1,20 thus represents a second unlinked locus, *VRN2*. 8A1,20 and 34C1,23 were found to be allelic. Since 8A1,20 and 34C1,23 were isolated from different M<sub>2</sub> families they represent independent alleles of the *VRN2* locus and have been designated *vrn2-1* and *vrn2-2*, respectively. The mutations 39A1,1 and 57D1,33 were not alleles of either *VRN1* or *VRN2* and thus represent at least one other independent locus (*VRN3*). For the crosses 34C1,23 × 47A1,7,7 and 39A1,1 × 47A1,7,7, the F<sub>2</sub> had  $\chi^2$  (9 early:7 late)  $P < 0.05$ , however, the large number of early plants segregating in both crosses clearly indicates that the mutations are not allelic. At the time the complementation tests were performed, other mutations induced by the EMS mutagenesis were segregating in the background, and it is possible that these mutations resulted in a distortion of the 9:7 F<sub>2</sub> ratio. All *vrn* mutations are being further backcrossed to *Ler* to eliminate such background mutations. The nomenclature of the mutant alleles is: 47A1,7,7 = *vrn1-1*; 8A1,20 = *vrn2-1*; 34C1,23 = *vrn2-2*; 57D1,33 = *vrn3-1*; 39A1,1-allelism not yet determined.

*vrn1-1* and *vrn2-1* show different responses to vernalization

The initial screens for *vrn* mutations were conducted under greenhouse conditions. In order to accurately assess the

**Table 2.** Dominance tests and complementation analyses—ratio of early to late flowering in F<sub>2</sub> progeny

Cross (early:late)	Segregation ratio	$\chi^2_{(3:1)}$	$\chi^2_{(9:7)}$
47A1,7,7 × <i>fca-1</i>	144:36	$P > 0.05$	-
8A1,20 × <i>fca-1</i>	143:37	$P > 0.05$	-
57D1,33 × <i>fca-1</i>	129:41	$P > 0.05$	-
39A1,1 × <i>fca-1</i>	126:34	$P > 0.05$	-
47A1,7,7 × 47A2,19	0:106	-	-
47A1,7,7 × 47A2,39	0:111	-	-
47A2,19 × 47A2,49	0:116	-	-
47A1,7,7 × 8A1,20	131:108	-	$P > 0.05$
47A2,19 × 8A1,20	70:50	-	$P > 0.05$
34C1,23 × 8A1,20	0:356	-	-
34C1,23 × 47A1,7,7	154:71	-	$P < 0.01$
57D1,33 × 8A1,20	141:96	-	$P > 0.05$
57D1,33 × 47A1,7,7	59:61	-	$P > 0.05$
39A1,1 × 8A1,20	164:124	-	$P > 0.05$
39A1,1 × 47A1,7,7	115:64	-	$P < 0.05$
57D1,33 × 39A1,1	n.d.	-	-

F<sub>2</sub> plants were vernalized for 8 weeks at 4°C and scored for segregation of early- to late-flowering plants. All *vrn* mutants have *fca-1* in their background. Plants having a leaf number greater than vernalized *fca-1* control plants were scored as late flowering. F<sub>2</sub> ratios with  $P > 0.05$  indicate segregation ratios consistent with either 3:1 or 9:7 early to late flowering. n.d., not determined.

**Table 3.** Total leaf number for vernalized and non-vernalized plants

Genotype	-Vernalization	+Vernalization
<i>Ler</i>	6.1 ± 0.1	5.9 ± 0.1
<i>Ler fca-1</i>	27.2 ± 1.0	8.1 ± 0.3
<i>vrn1-1 fca-1</i>	29.3 ± 1.6	17.0 ± 0.9
<i>vrn2-1 fca-1</i>	46.1 ± 2.6	31.8 ± 1.8

Numbers are means of total leaf numbers ± SE for eight to 20 plants.

flowering phenotype of *vrn1-1 fca-1* and *vrn2-1 fca-1*, the plants were grown under controlled conditions and the total leaf number (LN) at flowering was measured for vernalized or non-vernalized plants. The results are summarized in Table 3. In the absence of vernalization *vrn1-1 fca-1* plants flowered at the same time as *fca-1* plants, however, after a vernalization treatment they flowered considerably later. The *vrn1-1* mutation reduced the vernalization response of *fca-1* by 42%. *vrn2-1 fca-1* plants flowered later than *fca-1* plants in the absence of vernalization. After vernalization they flowered with 31 leaves, as late as *fca-1* without vernalization, suggesting that *vrn2-1* confers a complete loss of vernalization response. However, the later flowering of non-vernalized *vrn2-1 fca-1* plants implies that *vrn2-1* does not simply confer a complete loss of response to vernalization.

*The vrn mutations do not affect the acclimation response*

Cold temperature has a number of effects on the physiology of plants: in addition to causing earlier flowering, a cold treatment at 4°C for shorter periods of time than vernalization (up to 1 week) causes *Arabidopsis* plants to acclimate, and thus survive subsequent freezing temperatures (reviewed in Thomashow, 1994). Given the similarity of the initial environmental conditions necessary for both vernalization and acclimation we wanted to test whether the *vrn* mutants were impaired in their ability to acclimate. This was assayed in two ways, first, the induction of a number of transcripts previously shown to be strongly upregulated during an acclimation treatment was analysed. The expression of four cold-induced transcripts *COR15* (now designated *COR15a* (Thomashow, 1994)) *COR78*, *PHH7.2* and *PHH29* (Hajela *et al.*, 1990) was analysed following 4 days at 4°C. The results were similar for all four transcripts. Following acclimation, a very large increase in steady-state mRNA levels was seen in both *Ler* and *fca-1* plants following acclimation, which was indistinguishable from the increase seen in *vrn1-1 fca-1* and *vrn2-1 fca-1*.

The second assay was a freezing tolerance assay: *fca-1*, *vrn1-1 fca-1* and *vrn2-1 fca-1* plants non-acclimated or acclimated at 4°C for 1 week were subjected to progressively lower freezing temperatures. The temperature was reduced at 2°C h<sup>-1</sup>, the plants held at the final temperature for 6 h and then returned to 4°C with the same regime of increasing temperature. For all the genotypes, non-acclimated plants showed between 83–100% survival at -5°C but 0% survival at -6°C. For acclimated plants 85–100% of plants of all three genotypes survived temperatures down to -9°C (Jose Martinez-Zapater, personal communication).

Both assays thus demonstrate that the *vrn1-1* and *vrn2-1* mutations do not significantly impair the plant's acclimation response.

*The VRN1 locus maps to chromosome 3*

The chromosomal location of the *vrn1-1* mutation was determined by establishing linkage between the mutation and RFLP markers, either lambda clones (Chang *et al.*, 1988), or plasmid clones (provided by Robert Whittier, Mitsui Plant Biotechnology Research Institute, Japan). A homozygous *vrn1-1* plant was crossed to a line from the ecotype Wassilewskija (WS) carrying an *fca* mutation (a kind gift from R. Amasino, University of Wisconsin). F<sub>1</sub> plants were selfed to generate F<sub>2</sub> plants segregating for *vrn1-1* and RFLPs between *Ler* and WS ecotypes. Seed was collected from 77 F<sub>2</sub> plants. This seed was used for progeny testing for the *vrn* genotype, and to grow plants used to obtain DNA for RFLP analysis. Analysis of the

segregation pattern of 22 markers polymorphic between Landsberg *erecta* and WS indicated that *VRN1* mapped to the upper arm of chromosome 3. Finer analysis with markers mapping on chromosome 3 defined the interval in which *VRN1* mapped as being between *mi207* and *mi399*.

*vrn1-1 reduces the vernalization response of all the vernalization-responsive late-flowering mutants*

The *vrn1-1* mutation had no effect on the flowering time of non-vernalized *fca-1* and so appears to specifically disrupt the perception of, or response to, vernalization. It is also unlinked to the *FCA* locus and so could definitively be classified as a *vrn* mutation. It was of interest to see whether the *vrn1-1* mutation could disrupt the vernalization response of other vernalization-responsive, late-flowering mutants of *Arabidopsis*. In order to do this, it was necessary to segregate the *vrn1* mutation away from *fca-1*. A *vrn1-1 fca-1* homozygous plant was crossed to wild-type *Ler*, and individual early-flowering F<sub>2</sub> plants were selected. F<sub>3</sub> seed from selfed F<sub>2</sub> plants was collected and sown and the flowering time monitored in F<sub>3</sub> populations. Those F<sub>3</sub> populations not segregating any late-flowering plants represented progeny of F<sub>2</sub> plants which were homozygous for the wild-type *FCA* allele. Wild-type *Ler* plants only show a minimal vernalization response in long-day photoperiods, however, that response is much greater in short-days (mean LN non-vernalized approximately 28, mean leaf number vernalized approximately 22, Chandler, unpublished data). In order to select a line carrying the *vrn1* mutation, 20 F<sub>4</sub> seed from each F<sub>3</sub> plant homozygous for the wild-type *FCA* allele were vernalized, and grown under a short-day photoperiod. One F<sub>4</sub> family segregated later flowering individuals with a ratio of 3 early: 1 late. Late-flowering individuals were selected and selfed to confirm that they were indeed homozygous for the *vrn1-1* mutation.

This line was then backcrossed to *fca-1* and the flowering time after vernalization analysed in F<sub>2</sub> seedlings. Approximately 1/16 of the seedlings flowered late (Table 4). These experiments confirmed that we indeed had a line homozygous for the *vrn1-1* mutation that no longer carried the *fca-1* mutation.

This line was then crossed to the vernalization-responsive *Arabidopsis* late-flowering mutants *fve*, *ld*, *fwa*, *fe*, *fpa*, and *ft*. Segregation of flowering time, as assayed by LN, was analysed in vernalized F<sub>2</sub> progeny. The segregation ratio of early to late-flowering for each F<sub>2</sub> population is shown in Table 4. In all cases the ratio was not significantly different from 15:1, a ratio consistent with plants homozygous for both the late-flowering mutation and *vrn1-1* being late-flowering after vernalization. The whole experiment from the stage of crossing *vrn1-1* to the different late-flowering mutants was repeated and a similar set of ratios were found (data not shown).

**Table 4.** Segregation for flowering time in the F<sub>2</sub> from crosses of *vrn1-1* to late-flowering time in the F<sub>2</sub> from crosses of *vrn1-1* to late-flowering mutants

Cross	Segregation ratio (early:late)
<i>fca-1</i> × <i>vrn1-1</i>	164:14
<i>fve-1</i> × <i>vrn1-1</i>	172:7
<i>ld-3</i> × <i>vrn1-1</i>	156:12
<i>fwa-1</i> × <i>vrn1-1</i>	165:15
<i>fe-1</i> × <i>vrn1-1</i>	170:9
<i>fpa-2</i> × <i>vrn1-1</i>	170:10
<i>ft-1</i> × <i>vrn1-1</i>	168:12

Plants were scored as late if they flowered with more or equal to the rosette leaf number (LN) of the non-vernalized late-flowering mutant control plants. Non-vernalized controls flowered with mean LN; *fca-1* = 12.5, *fve-1* = 11, *fwa-1* = 11, *fe-1* = 12.5, *fpa-2* = 11, *ft-1* = 10.5, *Ler* = 5, *vrn1-1* = 8 and *vrn1-1 fca-1* = 12. Vernalized plants flowered with mean LN; *fca-1* = 6, *fve-1* = 5.5, *fwa-1* = 7.5, *fe-1* = 8, *fpa-2* = 6, *ft-1* = 7. In all cases the  $\chi^2$  value gave a probability of more than 0.1, indicating that the results are in agreement with those expected for a 15:1 ratio of early:late plants.

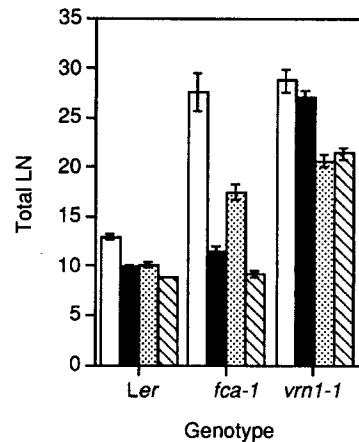
*Gibberellic acid still accelerates flowering time in vrn1 mutants*

We have previously reported that GA<sub>3</sub> application to plants grown in agar medium significantly accelerated the flowering time of all the late-flowering mutants (Chandler and Dean, 1994). Gibberellins have been implicated in the vernalization response of a number of plants (Bernier, 1988; Hazebroek and Metzger, 1990; Hazebroek *et al.*, 1993). Thus, we were interested to see whether the flowering time of the *vrn* mutants could also be accelerated by GA<sub>3</sub>. *Ler*, *fca-1* and *vrn1-1 fca-1* plants were grown in sterile conditions with or without vernalization and in addition with or without GA<sub>3</sub> application. Vernalization of *fca-1* caused a greater acceleration of flowering time (LN 27–12) than GA<sub>3</sub> treatment (LN 27–17). *vrn1-1* plants showed a much reduced response to vernalization but an almost wild-type response to GA<sub>3</sub>. These results indicate that the acceleration of flowering time by GA<sub>3</sub> is not impaired in *vrn1-1* mutants. When *fca-1* plants were given GA<sub>3</sub> and vernalized their flowering time was accelerated slightly compared with a vernalization treatment alone. This result suggests that GA<sub>3</sub> and vernalization may affect flowering time through different pathways. The flowering time of the *vrn1-1 fca-1* mutant in the double treatment was indistinguishable from that after a GA<sub>3</sub> treatment alone (Figure 2).

**Discussion**

*Identification of mutations affecting the vernalization response of Arabidopsis*

Through mutagenesis of the late-flowering *Arabidopsis* mutant *fca-1* we have identified three loci whose gene



**Figure 2.** The effect of gibberellic acid on vernalization of *Ler*, *fca* and *vrn1-1*. The four treatments shown for each genotype from left to right are: white, no vernalization no GA<sub>3</sub>; black, vernalization; stippled, GA<sub>3</sub>; hatched, vernalization plus GA<sub>3</sub>. Values are means ± SE for 12–18 plants.

products play a role in the vernalization response. The *vrn1* mutation reduces the effect of vernalization on flowering time without altering flowering time of non-vernalized *fca-1* plants. The *vrn2* mutation reduces the vernalization response but also causes a delay in flowering of non-vernalized *fca-1* plants. The other mutations have yet to be characterized in detail.

*The vernalization response exhibited by fca in LD and Ler in SD are both disrupted by vrn1*

The *vrn1-1* mutation reduces the vernalization response such that the plants flower with approximately half the number of leaves of non-vernalized *fca-1*. This still allows *vrn1-1* homozygotes to be easily scored in a segregating population. The fact that some response to vernalization is still seen may be explained by *vrn1-1* not being a null allele or by redundancy, either in *VRN1* function or in the whole vernalization pathway. What is clear, however, is that the *vrn1-1* mutation affects the vernalization response in wild-type Landsberg *erecta* and all the vernalization-responsive late-flowering mutants. This therefore implies that the vernalization pathway present in the late-flowering mutants and in short-day photoperiods is likely to be the same—mutations in the late-flowering loci probably just uncover a need for the vernalization-responsive pathway. Landsberg *erecta* plants grown in long-day photoperiods probably undergo a vernalization response but the presence of saturating floral promotive gene products means no effect of vernalization on flowering time is visible. Several models (Dennis *et al.*, 1996; Martinez-Zapater *et al.*, 1994; Weigel, 1995) have postulated that the vernalization pathway is normally only significant in long-day-grown plants when a pathway known as the 'constitutive pathway' which involves loci such as *FCA*, *FVE* and *LD*, is knocked out.

*vrn1 and vrn2 do not affect the acclimation response*

Neither *vrn1-1* or *vrn2-1* altered the acclimation response of *Arabidopsis*, monitored either through freezing tolerance or induction of cold-induced transcripts. We have not so far analysed whether the *vrn* mutations alter seed dormancy. If there is a single mechanism for sensing low temperature then the *vrn* mutations identify loci downstream in the pathway, specific to the vernalization response. Alternatively, acclimation and vernalization are two completely separate pathways, in which the perception of the cold temperature is possibly perceived in very different parts of the plant. In support of this, there is evidence that the vernalization signal is perceived at the apex and cannot be translocated from elsewhere in the plant (Metzger, 1988; Schwabe, 1954). It is likely that the acclimation signal is perceived throughout the plant or rapidly translocated.

*GA<sub>3</sub> still accelerates flowering in vrn1*

The acceleration of flowering by GA<sub>3</sub> was as great in *vrn1-1 fca-1* as in *fca-1*. Thus, either the acceleration of flowering time by vernalization and GA<sub>3</sub> act through different pathways or the influence of GA<sub>3</sub> is downstream of the point of action of the *VRN1* gene product. This result does not resolve the question of whether gibberellins are involved in the vernalization response. The *ga1-3* mutant, which carries a deletion in the *GA1* locus encoding a product necessary for the first committed step in GA biosynthesis (Sun and Kamiya, 1994), was found not to respond to vernalization in short-day photoperiods (Wilson *et al.*, 1992). Also, in *Thlaspi arvense*, a crucifer related to *Arabidopsis*, vernalization dramatically increased the hydroxylation of the GA precursor kaurenoic acid to 7-OH kaurenoic acid at the shoot tip through a direct effect on the KA hydroxylase enzyme (Hazebroek and Metzger, 1990; Hazebroek *et al.*, 1993). However, arguing against the fact that gibberellins are involved in vernalization in *Arabidopsis*, we have found that an *fca-1 ga1-3* double mutant grown in continuous days responds well to vernalization (Chandler and Dean, unpublished data).

The availability of *vrn* mutations opens up the possibility of looking at the interaction of vernalization with many other factors that affect flowering time, for example, far-red irradiation (Bagnall, 1993; Martinez-Zapater and Somerville, 1990). It also opens up the possibility of map-based cloning. An understanding of the mechanism of vernalization and how the vernalization pathway interacts with all the other pathways influencing flowering awaits the identification of all the genes involved in the perception of the vernalization response and analysis of the biochemical function of their gene products.

**Experimental procedures***Plant material and growth conditions*

Plants grown in soil were sown directly on to a mixture of soil:grit:vermiculite (3:2:2), in plastipak pots, and grown either in the greenhouse (temperature controlled at 20°C for 16 h during the day; 15°C at night; daylight extended with a light supplement of about 70 Wm<sup>-2</sup> from October until March), or in a Sanyo Gallenkamp controlled environment room under short-day or extended short-day growth conditions. Light conditions for the short-day room were 10 h illumination by 400 W Wotan metal halide power star lamps, PAR 113.7 μmol m<sup>-2</sup> sec<sup>-1</sup> and a R/FR ratio of 2.41. Light for the extended short-days was as for short-days (10 h) followed by 8 h illumination with Tungsten Halide lamps only, PAR 14.27 μmol m<sup>-2</sup> sec<sup>-1</sup>, and a R/FR ratio of 0.66. For plants grown on soil, individual plants were transferred to partitioned trays at about the four-leaf stage.

Plants grown in tissue culture were surface-sterilized by wetting with 70% ethanol, and soaking for 15 min in 5% (v/v) sodium hypochlorite with 0.2% Tween 20, followed by five rinses with sterile distilled water. Seeds were sown in petri dishes on AM media (1/2 MS salts (Flow labs); 0.5 mg l<sup>-1</sup> nicotinic acid; 0.5 mg l<sup>-1</sup> thiamine; 0.5 mg l<sup>-1</sup> pyridoxine; 100 mg l<sup>-1</sup> inositol; 0.8% agar; 1% sucrose), and grown at 20°C in white fluorescent light (PAR 57.0 μmol m<sup>-2</sup> sec<sup>-1</sup>; R/FR ratio 7.5; photoperiod 16 h). When first true leaves began to expand, plants were transferred individually to plastic capped boiling tubes (one plant per tube), in 9 × 4 racks (Sigma Ltd). Racks were placed in trays made of black card, to a depth of 2 cm, to shade the roots.

For gibberellic acid treatments, a stock solution of GA<sub>3</sub> was filter-sterilized and added to media in petri dishes and boiling tubes at a final concentration of 10<sup>-4</sup> M.

Plants were vernalized or acclimated at the seed stage, immediately after sowing on soil, or on agar. Vernalization was carried out for 8 weeks, and acclimation for 4 days, in an 8 h photoperiod (fluorescent light, PAR 9.5 μmol m<sup>-2</sup> sec<sup>-1</sup>, R/FR ratio 3.9) at a temperature of 5°C ± 1°C. Flowering time was assayed by total leaf number (LN), rosette plus cauline, counted once the bolt was more than 5 cm tall.

*Mutagenesis experiment*

A 0.5 g sample of *fca-1* seeds were soaked for 12 h in 0.3% ethyl methane sulphonate (EMS). Three thousand M<sub>1</sub> plants were grown in the greenhouse, and seed from 10 M<sub>1</sub> plants were bulk harvested to form M<sub>2</sub> families. One hundred and twenty plants from each of the 300 M<sub>2</sub> pools were vernalized on soil and grown in the greenhouse. Plants flowering with a greater LN than vernalized *fca-1* controls were selected as putative mutants having a decreased sensitivity to vernalization relative to *fca-1*.

*RFLP mapping*

For each F<sub>3</sub> family, DNA was isolated from 15–20 plants grown for 5 weeks in sterile liquid culture using a CTAB miniprep method (Dean *et al.*, 1992). Approximately 2 μg of genomic DNA were digested overnight with a fivefold excess of a restriction enzyme and fractionated by electrophoresis in 0.8% agarose gels at 0.5–2.0 Vcm<sup>-1</sup>. Gels were blotted and cross-linked to Hybond-N filters (Amersham) according to the manufacturer's instructions. Filters were prehybridized for 3–5 h and hybridized (10<sup>6</sup>–10<sup>7</sup> c.p.m. cm<sup>-3</sup>) for 16–18 h at 65°C in a solution containing 5× SSC (1×

SSC is 150 mM NaCl, 15 mM sodium citrate), 0.5% SDS, 5× Denhardt's solution (0.1% Ficoll (400), 0.1% PVP (360) and 0.1% BSA (fraction 5)), and denatured salmon sperm DNA (0.0025% w/v). Filters were washed at 65°C in 2× SSC for 5 min, and twice in 2× SSC containing 0.1% SDS for 30 min. Filters were exposed to Kodak X-Omat XAR X-ray film for 1–5 days at –70°C with an intensifying screen. Filters were re-used several times, after removing the previous probe by washing at 45°C in 0.4 M NaOH for 30 min followed by 15 min at 45°C in 0.1× SSC, 0.1% SDS and 0.2 M Tris–HCl pH 7.5.

Linkage analysis was performed on a Macintosh computer, using the MapMaker programme (Lander *et al.*, 1987), a gift from S.Tingey (DuPont Co.).

### RNA analysis

Total RNA was extracted using a method based on that of Logemann *et al.* (1987). Samples of between 0.5 and 3 g tissue were ground in liquid nitrogen using a mortar and pestle. Tissue was homogenized further by adding two volumes of guanidine buffer (8 M guanidine hydrochloride, 20 mM Mes, 20 mM EDTA and 50 mM mercaptoethanol at pH 7.0), and leaving for 1 h at room temperature. One volume of phenol/chloroform/isoamyl alcohol was added to the homogenate, which was then centrifuged at 1500 g for 10 min. The aqueous phase was collected and mixed with precooled 0.7 vol ethanol and 0.2 vol 1 M acetic acid and left overnight at –20° C. The precipitated RNA was recovered by pelleting at 1500 g for 10 min, and washed twice with sterile 3 M sodium acetate, pH 5.2 at room temperature, before centrifuging again at 1500 g for 5 min. The pellet was washed with 70% ethanol, and dissolved in 50–300 µl sterile water. RNA was denatured and separated by electrophoresis in formaldehyde-agarose gels and blotted according to Sambrook *et al.* (1989).

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