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Mutations causing defects in the biosynthesis and response to gibberellins, abscisic acid and phytochrome B do not inhibit vernalization in *Arabidopsis fca-1*

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Abstract. The roles of gibberellins, abscisic acid and phytochrome B in the vernalization response were investigated by combining mutations causing defects in their biosynthesis and response with the *Arabidopsis thaliana* (L.) Heynh. *fca-1* mutation. The *fca-1* mutation confers a very late-flowering phenotype which can be reversed to wild-type flowering if the seedlings are vernalized. Vernalization was unaffected in *ga1-3*, *gai*, *abi1-1*, *abi2-1*, *abi3-1* and *phyB-1* backgrounds, suggesting that gibberellin action mediated via *GA1* and *GAI*, abscisic acid action mediated through *ABI1* and *ABI2*, and phytochrome B, function independently of vernalization. However, the mutations did interact with *fca-1* to change flowering time in the absence of vernalization. The *abi1 fca-1* and *abi2 fca-1* double mutants flowered earlier than *fca-1* implying a role for abscisic acid in floral repression. Combination of *ga1-3* or *gai* with *fca-1* unexpectedly resulted in opposite interactions, with *gai* partially suppressing the late flowering of *fca-1*.

Key words: Abscisic acid – *Arabidopsis* (flowering time) – Gibberellin – Phytochrome B – Vernalization

Introduction

The use of a molecular-genetic approach in *Arabidopsis* has greatly improved our understanding of the control of flowering time. Analysis of the interactions of different flowering-time mutants has shown that multiple parallel pathways act to regulate the transition to flowering (reviewed in Koornneef et al. 1998; Levy and Dean 1998). Some of these pathways are promotive, some are repressive, some act to mediate environmental signals and others are involved in an endogenous timing mechanism. One floral promotion pathway, operating in both long (LD) and short day (SD) photoperiods, has been termed the autonomous promotion pathway and involves the genes, *FCA*, *FY*, *FVE* and *FPA* (Koornneef et al. 1991). Recessive mutations in these genes result in a late flowering phenotype which can be reversed to early flowering if the mutant seeds or seedlings are vernalized. The autonomous promotion and vernalization promotion pathways are therefore considered to be functionally redundant in the Landsberg *erecta* (L*er*) background in which the *fca*, *fy*, *fve* and *fpa* mutants were first isolated.

Gibberellins (GAs) have long been implicated in the control of flowering time and vernalization based on the fact that GA treatments can induce flowering and overcome a vernalization requirement in many species (Lang 1957 and reviewed in Pharis and King 1985; Levy and Dean 1998). In other species, however, GAs promote shoot extension but not flowering (Lang 1965). The requirement for GAs in flowering in *Arabidopsis* was demonstrated by examination of the flowering behaviour of *ga1-3* (Wilson et al. 1992). This mutant is completely deficient in *GA1* function which is required for the first committed step in GA biosynthesis. The mutant *ga1-3* was shown to be late flowering in LDs, did not flower in SDs and did not respond to vernalization in the SD conditions. Gibberellin has also been shown to activate the transcription of *LEAFY*, a gene that is both necessary and sufficient to cause the floral transition in *Arabidopsis* (Weigel and Nilsson 1995; Blázquez et al. 1998). In *Thlaspi arvense*, vernalization has been proposed to specifically induce the synthesis of GA_9 which then promotes stem elongation (Metzger 1988; Hazebroek and Metzger 1990; Hazebroek et al. 1993). This model was then extended to propose that vernalization caused methylation changes at the gene encoding kaurenoic acid hydroxylase, the enzyme that converts kaurenoic acid to 7-OH kaurenoic

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Abbreviations: ABA = abscisic acid; GA = gibberellin; L*er* = Landsberg *erecta*; LD = long day; SD = short day

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acid – GA_9 (Burn et al. 1993; Finnegan et al. 1996, 1998). Other studies do not support vernalization inducing specific GAs. For example, GA composition was found to be similar in vernalized and non-vernalized winter canola shoot tips, with vernalization potentially altering GA metabolism (Zanewich and Rood 1995).

Phytochrome B mediates in the promotion of flowering by far-red (FR)-enriched light or end-of-day FR treatments (Nagatani et al. 1991; Bagnall et al. 1995). *Arabidopsis* mutants or ecotypes showing the strongest response to increased FR light also show the strongest vernalization response (Martinez-Zapater and Somerville 1990; Bagnall 1992), potentially implicating phytochrome B function in the vernalization response. Phytochrome B has been proposed to decrease both GA biosynthesis and responsiveness to GAs (Rood et al. 1990; Beall et al. 1991; Devlin et al. 1992; Xu et al. 1995; Reed et al. 1996; Yamaguchi et al. 1998) so the phytochrome effects on flowering/vernalization may act through differential GA biosynthesis and sensitivity. Koornneef et al. (1995) suggested that active phytochrome B may inhibit an *FCA*-dependent GA action necessary for flowering in SDs.

The role for abscisic acid (ABA) in flowering has been studied much less. Many aspects of plant growth and development as well as response to low temperature conditions are regulated by ABA (Chandler and Robertson 1994) which often acts as an antagonist to GA action (Jacobsen et al. 1995). Mutants insensitive to ABA (*abi*) were reported to flower earlier in SDs (Martinez-Zapater et al. 1994) and ABA has been found to both promote and inhibit the flowering of *Pharbitis nil* depending on when after the light stimulus the ABA is applied (Bernier 1988; Takeno and Maeda 1996).

In order to further investigate the roles of GAs, ABA and phytochrome B in vernalization, we have generated double mutants between *fca-1* and mutations in either the biosynthesis or response to GAs, ABA and phytochrome B. The strong *fca-1* allele carries a single nucleotide substitution that results in a premature translation stop codon and so is likely to cause a complete loss of function (Macknight et al. 1997). Here we report the characterization of these mutants in terms of flowering time and vernalization response.

Materials and methods

Plant genotypes and generation of double mutants. All the mutations described in this paper are derived from *Arabidopsis thaliana* (L.) Heynh., ecotype Landsberg *erecta*, and were obtained from Prof. M. Koornneef (Department of Genetics, Wageningen University). The late-flowering, vernalization-responsive *fca-1* mutation was induced by ethyl methanesulfonate (EMS; Koornneef et al. 1991). The GA-insensitive mutation (*gai*) was induced by X-ray irradiation (Koornneef et al. 1985), and the GA-deficient mutant, *ga1-3*, by fast neutron irradiation (Koornneef et al. 1983). The long-hypocotyl mutant *phyB-1* (originally named *hy3-1*) and the ABA-insensitive mutants *abi1-1*, *abi2-1* and *abi3-1* were all EMS-induced (Koornneef et al. 1984). F_2 populations of different crosses were grown in petri dishes on AM medium (1/2 MS salts; 0.5 mg l^{-1} nicotinic acid; 5 mg l^{-1} thymidine; 0.5 mg l^{-1} pyrridoxine; 100 mg l^{-1} inositol; 0.8% agar; 1% sucrose). For crosses with *abi1-1*, *abi2-1* and *abi3-1*, 10 μM ABA was included in the growth medium. Double mutants germinated at this concentration of ABA and were later flowering than the ABA-insensitive mutant following transferral of plants to soil after 2 weeks. For the cross with *ga1-3*, plants failing to germinate on AM medium alone were transferred after 10 d to medium containing 0.1 μM gibberellic acid (GA_3) and late-flowering individuals were then selected as the double mutant. For crosses with *gai* and *phyB-1*, the plants showing these mutations were selected, and transferred to soil after 2 weeks in sterile culture, and late-flowering plants selected. All double mutants were confirmed by progeny testing.

Growth conditions. The *ga1-3* and *gai* single and double mutants were grown at 20 °C in an extended SD cabinet [10 h photosynthetically active radiation (PAR) 92.9 μmol m^{-2} s^{-1} red (R):FR 1.49, 6 h PAR 14.27 μmol m^{-2} s^{-1} R:FR 0.66]. The repeated *gai fca-1* experiment and the *phyB* and *abi* single and double mutants were grown in continuous light from fluorescent lamps (PAR 34.6 μmol m^{-2} s^{-1}, R/FR ratio 6.1) at 20 °C. Leaf number (LN), which has been shown to be highly correlated with flowering time (FT) (Koornneef et al. 1991) was used as the measure of flowering time, and was scored as the total number of rosette and cauline leaves. Seeds of the *ga1-3* and *fca-1*, *ga1-3* mutants require exogenous GA to germinate. Therefore, seeds were soaked in 0.1 mM GA_3 (approximately 1 cm^3 per 100 seed) in a plastic capped tube for 2 d in the growth chamber, and then rinsed with four 10-cm^3 changes of water before sowing directly onto soil. No difference in flowering time was observed between treated and untreated plants, showing that the GA_3 treatment used was enough to effect germination of the *ga1-3* and *ga1-3 fca* mutants, without affecting subsequent LN at flowering. Pots containing seeds to be vernalized were wrapped in clingfilm and kept in the growth chamber for 24 h before the vernalization. After vernalization, pots were transferred back to the growth chamber, and the clingfilm removed. When plants had reached about the four-leaf stage, they were individually transferred to partitioned trays at a density of approximately one plant per 10 cm^3. For each experiment, the mutant, double mutant with *fca-1*, wild type and *fca-1* were all grown simultaneously.

Vernalization was carried out for 8 weeks at 5 °C ± 1 °C in an 8-h photoperiod under fluorescent lamps (PAR 9.5 μmol m^{-2} s^{-1}, R/FR ratio 3.9). In the *phyB-1*, *abi1-1*, *abi2-1* and *abi3-1* experiments, plants were vernalized for 1, 2, 4, 7, 11, 16, 22, 28, 42 or 56 d before transferring back to the growth chamber. For the *ga1-3 gai* experiment, plants were vernalized for 7, 14, 21, 42 or 56 d before transferring back to the growth chamber.

Results

Combination of fca-1 with mutations affecting GA biosynthesis and response. The *GA1* gene encodes ent-kaurene synthase, the first committed step of GA biosynthesis. The *ga1-3* allele, which carries a deletion removing a major portion of the coding region (Sun et al. 1992) reduces GA levels to <1% of wild-type levels (Zeevaart and Talón 1992). In order to test the requirement for GA biosynthesis during vernalization, double mutants *ga1-3 fca-1* were generated. These mutants together with L*er*, *fca-1* and *ga1-3* plants were subjected to varying doses of vernalization, then returned to the growth chamber and flowering time assayed by counting leaf number.

In agreement with previous work, the *ga1-3* mutant flowered later than its wild-type L*er* parent (Fig. 1A) (Wilson et al. 1992). Eight weeks of vernalization did not significantly alter the flowering time of L*er* and *ga1-3* plants. However, *fca-1* plants responded strongly

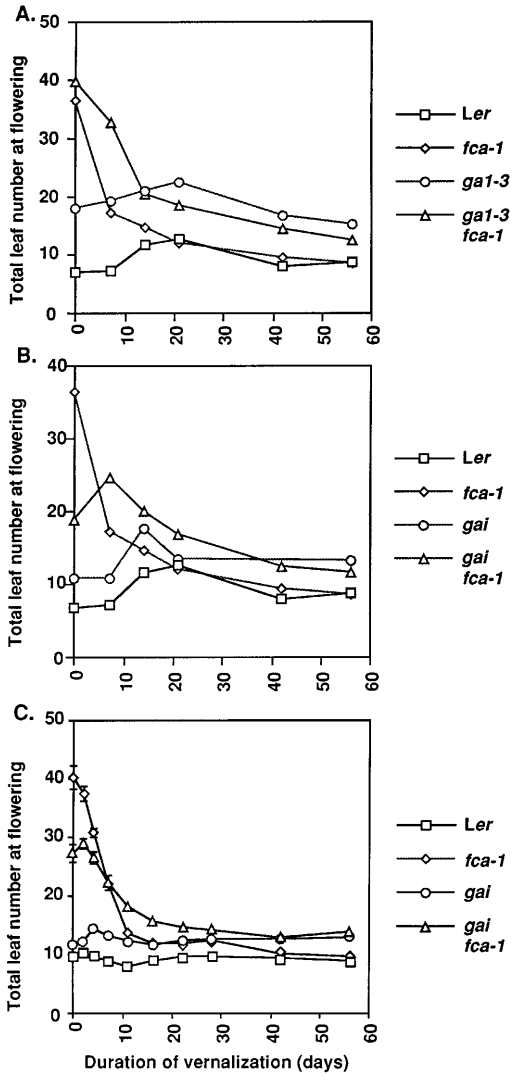

Fig. 1A–C. The effect of vernalization on leaf number of L*er* and *fca-1* and either *ga1-3*, *ga1-3 fca-1* (**A**) or *gai* and *gai fca-1* (**B, C**). Plants used in **A** and **B** were grown in an extended-SD cabinet, whereas those in **C** were grown in continuous light from fluorescent lamps. Points are means ±SE for 8–28 plants. For most data points, SE bars are obscured by the symbols

to vernalization with the leaf number being reduced by half after only one week of cold treatment. After 8 weeks vernalization, the leaf number of *fca-1* plants was the same as that of the L*er* parent. The *ga1-3 fca-1* double-mutant plants flowered significantly later than the *fca-1* parent. The *ga1-3 fca-1* double-mutant, however, still responded strongly to vernalization. The vernalization dose response curve for *ga1-3 fca-1* double-mutant plants followed that for *fca-1* except that the double mutants flowered with about five extra leaves at every time point. After 8 weeks vernalization the *ga1-3 fca-1* double mutant flowered with a leaf number similar to that of *ga1-3*.

The same kind of experiment was conducted for *gai fca-1* plants (Fig. 1B). Without vernalization, the *gai fca-1* double mutants flowered with approximately half the number of leaves of *fca-1*. This contrasts with the delay in flowering observed when *ga1-3* was combined with *fca-1*. The *gai* and *ga1-3* mutant plants generally show the same phenotypic characteristics. However, in combination with *fca-1*, the GA-biosynthesis and GA-response mutants showed very different interactions. A 7-d vernalization period delayed flowering of *gai fca-1*, *gai* and L*er*, although the delay in the latter two occurred after 14- or 21-d vernalization periods. After 8 weeks of vernalization, *gai fca-1* plants flowered at the same time as the *gai* parent.

Given the unexpected interaction between *gai* and *fca-1*, the analysis with *gai* single and double mutants was repeated using the conditions used for the *phyB* and *abi* mutants (Fig. 1C). The earlier flowering of *gai fca-1* compared to *fca-1* was reproduced together with the slight delay in flowering caused by short vernalization times. In this experiment, however, the delay occurred in plants vernalized for between 2 and 4 d rather than 14–21 d.

A third experiment on *gai* and *fca-1* single- and double-mutant seedlings, grown in the same conditions as those shown in Fig. 1B, gave total leaf number values of *gai* 11.04 ± 0.24; *fca-1* 35 ± 0.8; *gai fca-1* 21.3 ± 0.42.

Combination of fca-1 with mutations affecting ABA response. Double-mutant combinations of *fca-1* with *abi1-1*, *abi2-1* and *abi3-1* were obtained. Flowering time following varying amounts of vernalization was assayed for single-mutant parents and the *abi1-1 fca-1*, *abi2-1 fca-1* and *abi3-1 fca-1* double mutants. The results for *abi1-1* and *abi2-1* were very similar and only those for *abi2-1* are shown (Fig. 2A). The *abi2-1* mutant flowered at the same time as the L*er* parent and this was unaffected by vernalization. The *abi2-1 fca-1* double mutant flowered earlier than *fca-1* with approximately 10 fewer leaves. Vernalization reduced the leaf number at flowering such that the *abi2-1 fca-1* double mutant had the same leaf number as the L*er* parent after 15 d of vernalization. The acceleration of flowering by different periods of vernalization was similar for *abi2-1 fca-1* and *fca-1*. Therefore, *abi1-1* and *abi2-1* mutations significantly accelerate the flowering of *fca-1* without affecting the vernalization response.

The *abi3-1 fca-1* double mutant showed the same rate and degree of vernalization response as *fca-1* for the first 7 d. The degree of response after 56 d was also the same. However, for vernalization periods of between 1 and 6 weeks duration, the double mutant had more leaves than *fca-1* (Fig. 2B). It would appear therefore that unlike *abi1* and *abi2*, *abi3* does reduce the effectiveness of intermediate vernalization periods.

The effect of phyB-1 on flowering and vernalization of fca-1. The *phyB-1 fca-1* double mutant was identified and then sown alongside the single-mutant parent after varying days of vernalization. The *phyB-1* mutant flowered significantly earlier than the L*er* wild type (agreeing with Koornneef et al. 1995), and leaf number at flowering was not affected by vernalization (Fig. 3). The *phyB-1 fca-1* double mutant had significantly fewer leaves than *fca-1* in the absence of vernalization (Fig. 3), but

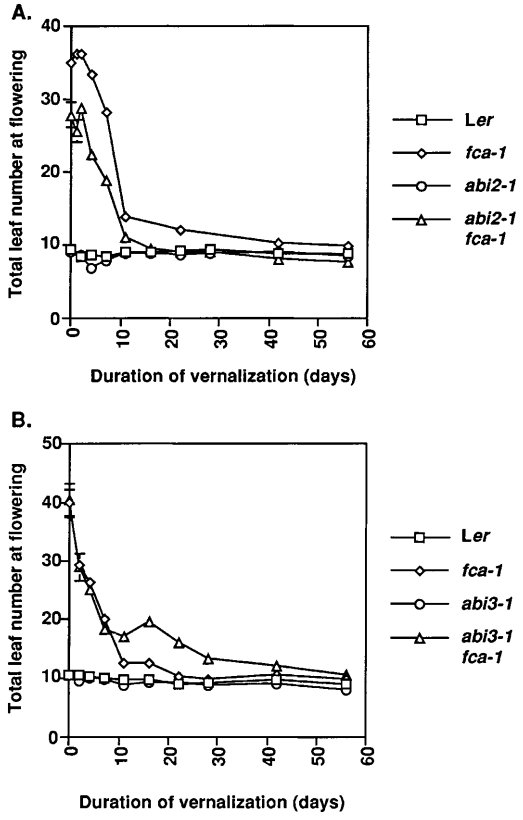

Fig. 2A–B. The effect of vernalization on leaf number of L*er* and *fca-1* and either *abi2*, *abi2 fca-1* (**A**) or *abi3* and *abi3 fca-1* (**B**). Points are means ±SE for 10–18 plants. For most data points, SE bars are obscured by the symbols

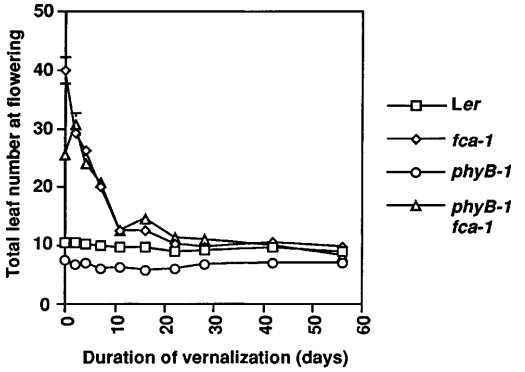

Fig. 3. The effect of vernalization on leaf number of L*er*, *fca-1*, *phyB-1* and *phyB-1 fca-1*. Points are means ± SE for 8–18 plants. For most data points, SE bars are obscured by the symbols

showed the same rate and extent of vernalization response for all vernalization treatments greater than 2 d.

Discussion

The aim of this study was to investigate the role of GAs, ABA and phytochrome B in the vernalization response. However, most of the interactions detected addressed their role in floral pathways other than vernalization. The *fca-1 ga1-3* double-mutant plants flowered significantly later than *fca-1*, suggesting that *GA1* and *FCA* promote the floral transition through independent pathways. Introduction of *ga1-3* and *gai* into a *co-2* background, a late-flowering mutant in the photoperiodic pathway (Putterill et al. 1995) resulted in very late flowering in both cases. It was therefore surprising that the *gai fca-1* double mutant flowered earlier than *fca-1*. This earliness, however, is not a feature of all mutants of the autonomous promotion pathway as when *gai* is combined with *fve-2*, the double mutant flowers very much later than the *fve* parent (L. Ruiz-Garcia and J. Martinez-Zapater, CNB-CSIC, Madrid, personal communication). The basis of the earliness of *gai fca-1* and why *fca-1* shows a different interaction with *gai* than with *ga1-3* remains to be established. Endogenous GA levels are significantly higher in *gai* mutant plants than the wild type (Talon et al. 1990) but it is not clear if this would contribute to the earliness given the late flowering of *gai* itself. A preliminary observation that leaf production rate in *gai fca-1* may be faster than in *gai* could be part of the explanation (data not shown).

The *abi1-1* and *abi2-1* mutations significantly accelerated the flowering of *fca-1*; however, the repressive action of ABA on flowering in LDs is only detected in an *fca-1* background as *abi1-1* and *abi2-1* did not flower later then L*er*. The *phyB-1* mutation accelerated the flowering of *fca-1* as reported previously (Koornneef et al. 1995) showing that phytochrome represses the floral transition even in the absence of *FCA* function.

There has been much speculation about the role of GAs in vernalization. Our results would indicate that the vernalization response was almost complete in a *ga1-3* mutant. The analysis of the vernalization response of *ga1-3* undertaken by Wilson et al. (1992) could only study vernalization under SDs as in their continuous light conditions the *ga1-3* mutant flowered at the same time as wild-type plants. Under SD conditions, they found that vernalization did not accelerate the flowering of *ga1-3*. This could have implied a role for GAs in vernalization but in light of the findings described here, the results suggest that even after vernalization, GAs are required for flowering in SDs. Our results indicate that GAs are not required for vernalization but we cannot completely rule out their participation as small amounts of several GAs have been detected in *ga1-3* seedlings, suggesting the presence of genes that can function partially redundantly with *GA1* (Zeevaart and Talón 1992).

The *abi1-1* and *abi2-1* mutations had no effect on the vernalization response of *fca-1*. This would support the idea that ABA does not play a major role in vernalization. In the *abi3 fca-1* double mutant the effectiveness of intermediate vernalization periods appeared to be reduced. The major function of *ABI3* is in controlling seed development (Finkelstein and Somerville 1990) and this is in agreement with the lack of effect in the non-vernalized double mutant. However, null alleles of *abi3-1* result in a range of phenotypes that suggest *ABI3* may have broader functions outside ABA signal transduction (Bonetta and McCourt 1998). The effect seen on vernalization here supports this argument. Lack of phytochrome B does not seem to enhance or impair

vernalization as after 2 d of vernalization the acceleration of flowering by vernalization in the *phyB-1 fca-1* plants followed the curve for *fca-1*. If GA biosynthesis or sensitivity is altered in *phyB* mutants this is not impacting on the vernalization response.

Further understanding of the processes involved in vernalization will need a detailed analysis of mutants that show a reduced vernalization response. Such *vrn* mutants have been isolated in an *fca-1* background (Chandler et al. 1996). Their interaction with mutations in different hormone and light signalling pathways and the cloning of the *VRN* genes should begin to provide insight into the molecular processes involved in vernalization. Similarly, combined genetic and molecular analysis should reveal how *GA1*, *GAI*, *ABI1*, *ABI2* and *PHYB* interact with the *FCA*-mediated floral promotion pathway.

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