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Gibberellins Promote Flowering of Arabidopsis by Activating the *LEAFY* Promoter

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The gibberellin class of plant hormones has been implicated in the control of flowering in several species. In Arabidopsis, severe reduction of endogenous gibberellins delays flowering in long days and prevents flowering in short days. We have investigated how the differential effects of gibberellins on flowering correlate with expression of *LEAFY*, a floral meristem identity gene. We have found that the failure of gibberellin-deficient *ga1-3* mutants to flower in short days was paralleled by the absence of *LEAFY* promoter induction. A causal connection between these two events was confirmed by the ability of a constitutively expressed *LEAFY* transgene to restore flowering to *ga1-3* mutants in short days. In contrast to short days, impairment of gibberellin biosynthesis caused merely a reduction of *LEAFY* expression when plants were grown in long days or with sucrose in the dark. As a first step toward identifying other small molecules that might regulate flowering, we have developed a rapid in vitro assay for *LEAFY* promoter activity.

INTRODUCTION

The most dramatic phase change that flowering plants undergo is the transition from vegetative to reproductive growth. For this transition to be successful, plants must integrate a variety of environmental signals with endogenous cues, such as plant age (Bernier, 1988).

In the facultative long-day plant Arabidopsis, the transition to reproductive growth occurs rapidly in long days but much more slowly in short days. Several flowering-time mutants, in which the timing of this transition is changed, have been isolated. Analysis of the responses of different mutants to the environment together with studies of their genetic interactions have resulted in a two-pathway model showing how the transition to flowering is regulated (Martínez-Zapater et al., 1994; Weigel, 1995; Peeters and Koornneef, 1996). According to this model, long days induce flowering via a facultative and fast pathway, whereas under noninductive photoperiods, an autonomous and much slower pathway is rate limiting. The latter pathway is thought to be related to plant age.

The gibberellin (GA) class of plant hormones plays a role in many processes during plant development, including seed germination, cell elongation, and flowering (Finkelstein and Zeevaart, 1994). In Arabidopsis, physiological and genetic experiments have implicated GAs specifically in the autonomous pathway of flowering. Exogenous application of GAs accelerates flowering in wild-type Arabidopsis, particularly in short days (Langridge, 1957). That there is a causal connection between endogenous GA levels and flowering in Arabidopsis has been confirmed with several GA biosynthesis and signaling mutants. Mutants in which GA levels are severely reduced, such as *ga1-3*, are unable to flower in short days (Wilson et al., 1992). *ga1-3* mutants carry a deletion of the gene encoding *ent*-copalyl diphosphate synthase (formerly *ent*-kaurene synthetase A), which controls a key step in early GA biosynthesis (Koornneef and Van der Veen, 1980; Zeevaart and Talón, 1992; Sun and Kamiya, 1994). In long days, flowering of *ga1-3* mutants is only slightly delayed when compared with the wild type, indicating partial redundancy of the pathway involving *GA1* under these conditions (Wilson et al., 1992; Silverstone et al., 1997).

Factors that can at least partially substitute for *GA1* activity in long days include the *CONSTANS* (*CO*) gene, which promotes flowering in response to long days. Consistent with the redundancy of the *CO* and *GA1* pathways, double mutants that carry the *ga1-3* allele along with a mutation in *co* often do not flower at all in long days (Putterill et al., 1995). A phenotype similar to that of *ga1-3* is seen in the GA-insensitive *gai* mutant, which flowers considerably later than does the wild type in short days (Koornneef et al., 1985; Wilson et al., 1992). In contrast, mutations at the *SPINDLY* (*SPY*) locus constitutively activate GA signal transduction, and *spy* mutants flower early (Jacobsen and Olszewski, 1993).

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The transition to flowering involves a change in the identity of the primordia arising at the flanks of the shoot apical meristem from leaves with associated lateral shoots (paraclades) to bractless flowers. This switch is dependent on the activity of floral meristem identity genes, such as *LEAFY* (*LFY*) and *APETALA1* (reviewed in Yanofsky, 1995). Both genes are expressed at high levels in emerging flower primordia, but only *LFY* is also expressed in leaf primordia before the transition to flowering is made (Blázquez et al., 1997; Hempel et al., 1997). The level of *LFY* expression in the primordia produced by the shoot apical meristem increases with the age of the plant until it apparently reaches a threshold level. Once this level has been reached, a primordium that would otherwise turn into a leaf/paraclade becomes a bractless flower instead. That additional copies of wild-type *LFY* cause plants to produce fewer leaves before the first flower is formed confirms that *LFY* expression levels are critical for the fate switch from leaf/paraclade to bractless flower (Blázquez et al., 1997).

A question that emerges from these observations is how expression and activity of floral meristem identity genes are controlled. Here, we describe experiments that link the effect of GAs on flowering to *LFY* transcription and activity. We show that *LFY* promoter activity is reduced in mutants defective in GA biosynthesis and that the failure of *ga1-3* mutants to flower in short days can be overcome by constitutive expression of *LFY.* Conversely, constitutive GA signaling in *spy* mutants causes an increase in *LFY* promoter activity. These and other results suggest that GAs affect flowering through a pathway that controls *LFY* transcription.

RESULTS

Reduction of *LFY* Promoter Activity in GA-Deficient Mutants

Exogenous application of GA_3 accelerates flowering in short days, and this is paralleled by an increase in *LFY* promoter activity (Langridge, 1957; Wilson et al., 1992; Blázquez et al., 1997). To test whether these effects reflect a role for endogenous GAs in controlling *LFY* promoter activity, we introduced a *LFY*::β-glucuronidase (*GUS*) transgene into *ga1-3* mutants. We have shown previously that this transgene closely mimics the expression of endogenous *LFY* RNA during both vegetative and reproductive development (Blázquez et al., 1997). The *ga1-3* mutants carry a deletion of the *GA1* locus and have severely reduced endogenous GA levels (Wilson et al., 1992; Zeevaart and Talón, 1992; Sun and Kamiya, 1994).

Similar to previous reports (Silverstone et al., 1997), we found that *ga1-3* mutants flowered in long days later than did the wild type and had 16 rather than 11 leaves (Table 1). The delay in the transition to flowering was paralleled by a change in the profile of LFY::GUS activity, which was affected in three different ways. First, initial levels of LFY::GUS activity were reduced; second, the subsequent upregulation of LFY::GUS was delayed; third, the maximal level of LFY::GUS activity was much lower (Figure 1A). To confirm that the initial reduction in *LFY*::*GUS* levels was significant, we analyzed LFY::GUS activity in 4-day-old seedlings by histochemical staining with X-gluc. LFY::GUS activity is easily detectable in the first two leaf primordia of wild-type seedlings immediately after germination (Blázquez et al., 1997). In contrast, approximately three-quarters of *ga1-3* seedlings did not show any staining, whereas the remainder showed only weak staining (data not shown). Treatment of *ga1-3* mutants with exogenous GA_3 restored LFY::GUS activity to a nearly wild-type profile (Figure 1A), with the small difference probably being a consequence of the treatment starting only after day 4. Wild-type plants showed little response to exogenous GA_3, which is consistent with GA_3 having only a very modest effect on flowering time of long-day-grown wild-type plants.

As reported previously (Wilson et al., 1992), *ga1-3* mutants never flowered in short days unless treated with GA_3. The failure to flower was paralleled by absence of significant *LFY*::*GUS* upregulation during the 3 months of the experimental period (Figure 1B). This result was confirmed visually

Table 1. Flowering Time of the *ga1* and *spy* Mutant Lines Used in This Study

		Long Days[b]			Short Days[b]		
Line[a]	Genotype	RL	CL	TL	RL	CL	TL
150-307	Wild type	9.0 ± 0.2	2.4 ± 0.1	11.4 ± 0.2	27 ± 1	8 ± 1	35 ± 1
150-304	Wild type	9.1 ± 0.1	2.5 ± 0.2	11.6 ± 0.2	26 ± 1	8 ± 1	34 ± 1
307G1	*ga1-3*	—[c]	—[c]	15.7 ± 0.6	>55	—[d]	>55
304Y5	*spy-5*	7.1 ± 0.2	3.2 ± 0.2	10.3 ± 0.3	22 ± 1	6 ± 1	28 ± 1

[a] All lines are in the Landsberg *erecta* background and homozygous for the *LFY*::*GUS* transgene.

[b] RL, rosette leaves; CL, cauline leaves; TL, total number of leaves. Measurements are the mean ±2 × SE of the mean ($n \geq 12$ plants).

[c] (−) indicates that *ga1-3* plants did not bolt in long days, and cauline leaves could not be differentiated from rosette leaves.

[d] (−) indicates that *ga1-3* plants did not flower in short days.

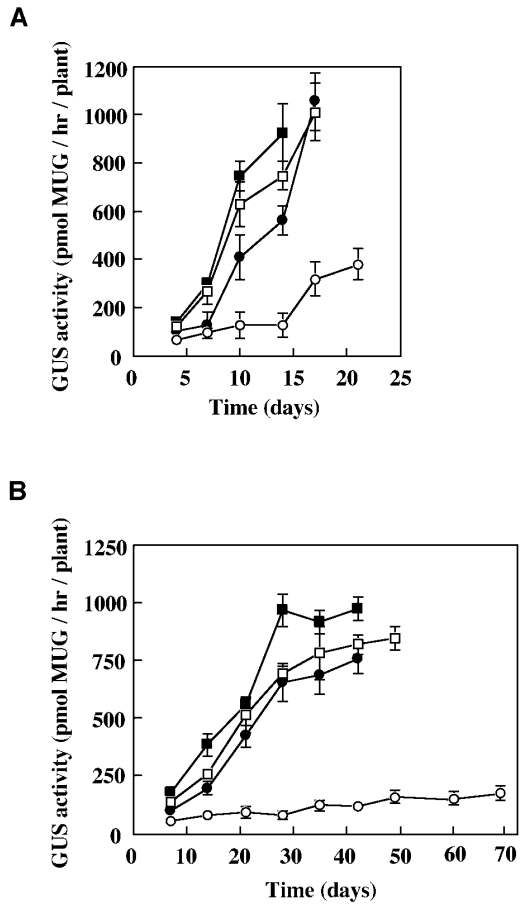

Figure 1. *LFY*::*GUS* Expression during Vegetative Growth of *ga1-3* Mutants.

(A) LFY::GUS activity in long days. Plants homozygous for the *LFY*::*GUS* transgene in a *GA1*$^+$ (DW150-307; squares) or *ga1-3* (307G1; circles) background were grown in long days until flower buds were visible to the naked eye. Measurements from plants treated with GA_3 are indicated by filled symbols, and those from untreated plants by open symbols.

(B) LFY::GUS activity in short days. Symbols are the same as given for **(A)**. Plants were grown in short days until flower buds were visible to the unaided eye, except in the case of the *ga1-3* mutant without GA_3 treatment (open circles), which had not flowered at the end of the experiment.

Values are expressed as mean $\pm 2 \times$ SE of the mean ($n \geq 10$). Time represents days after sowing. Error bars that are not visible are smaller than the graph symbol. MUG, 4-methylumbelliferyl β-D-glucuronide.

by histochemical staining of young seedlings. GUS activity could be detected easily in young leaves of wild-type seedlings grown in short days (Figure 2A), whereas *ga1-3* mutants grown in the same conditions did not show any staining (Figure 2B). As occurs in long days, exogenous application of GA_3 largely rescued both the flowering defect and *LFY*::*GUS* expression in *ga1-3* mutants (Figure 1B). As reported previously, GA_3 application accelerated LFY::GUS upregulation and flowering in wild-type plants (Wilson et al., 1992; Blázquez et al., 1997). To confirm that expression of the endogenous *LFY* gene, and not only that of *LFY*::*GUS*, was severely reduced in the *ga1-3* mutant, RNA expression was analyzed by reverse transcription followed by polymerase chain reaction (RT-PCR). Although *LFY* RNA could be detected in the apices of both 4-week-old wild-type and *ga1-3* plants grown in short days, *LFY* RNA levels were ~10 times lower in *ga1-3* mutants when compared with the wild type (Figures 2A and 2B).

Besides mutations, pharmacological agents can be used to inhibit the GA pathway in plants. Paclobutrazol interferes with GA biosynthesis by preventing the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher, 1991), and we found that the effect of *ga1-3* on flowering time and on *LFY*::*GUS* expression could be mimicked by treating wild-type plants with a solution of 12.5 mg/L paclobutrazol (data not shown).

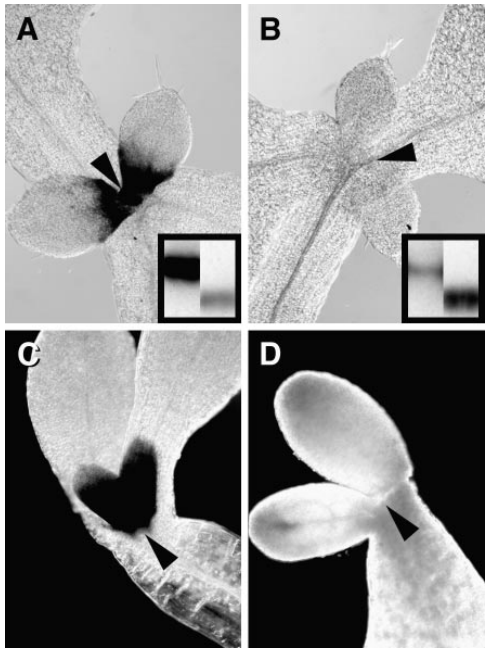

Figure 2. Histochemical Localization of GUS Activity in Seedlings Carrying a *LFY*::*GUS* Transgene.

GUS activity is visible as a dark precipitate. The positions of the shoot apical meristems are indicated by black arrowheads. Two true leaves are visible in their entirety in the short-day-grown plants (**[A]** and **[B]**), and the cotyledons are visible in the dark-grown plants (**[C]** and **[D]**). The insets show *LFY* (left) and *eIF4A* (right) RNAs detected by RT-PCR in apices of 4-week-old plants (**[A]** and **[B]**).

(A) Ten-day-old wild-type seedling grown in short days.

(B) Ten-day-old *ga1-3* seedling grown in short days.

(C) Seven-day-old wild-type seedling grown in darkness.

(D) Seven-day-old *ga1-3* seedling grown in darkness.

It has been proposed that flowering in short days depends on an environment-independent pathway promoting flowering (Martínez-Zapater et al., 1994). If this model is correct, *LFY* promoter activity under photoperiods that are shorter than the critical length for early flowering might not depend on ambient light conditions at all. To test this idea, we examined *LFY*::*GUS* expression in dark-grown plants. Wild-type Arabidopsis plants can develop and flower in the dark, provided that the aerial part of the plant is in contact with sucrose-containing medium (Araki and Komeda, 1993; Roldán et al., 1997). This effect can be achieved by growing plants on a vertical substrate consisting of solid Murashige and Skoog (MS; Murashige and Skoog, 1962) medium and sucrose (Roldán et al., 1997). When we cultured wild-type plants under these conditions, they produced six or seven leaves before flowering.

Similar to the pattern seen in plants grown in short days, we detected GUS activity in young leaf primordia of dark-grown *LFY*::*GUS* plants (Figure 2C), indicating that flowering in the dark also correlates with *LFY* expression. To determine the contribution of GAs, we examined *ga1-3 LFY*::*GUS* plants grown in the dark. *ga1-3* mutants developed as etiolated dwarves but eventually flowered, albeit with a significant delay compared with wild-type plants (with 20 to 25 leaves) (Roldán et al., 1997). Interestingly, the *ga1-3* mutation abolished *LFY*::*GUS* expression in young seedlings (Figure 2D). However, LFY::GUS activity could be detected in leaf primordia after 15 leaves had been produced (data not shown). These results indicate either that GAs are less important for flowering in the dark than in short days or that sucrose in the culture medium can compensate for low GA levels in *ga1-3* mutants.

Increase in *LFY* Promoter Activity in Mutants with an Elevated Response to GA

To obtain further evidence that endogenous GA signaling modulates *LFY* expression, we introduced the *LFY*::*GUS* transgene into plants carrying a lesion in *SPY*, a negative regulator of GA signaling (Jacobsen and Olszewski, 1993). *spy-5 LFY*::*GUS* plants were grown in long and short days and assayed for GUS activity during the vegetative phase. Only in short days did *spy-5* mutants flower significantly earlier than did wild-type plants (28 versus 34 leaves; Table 1). Consistent with the absence of a large effect in long days, *LFY*::*GUS* expression in *spy-5* mutants was only slightly increased in long days (Figure 3A). The difference between the mutant and the wild type was more accentuated in short days (Figure 3B), resembling the effect of applying GA_3 to wild-type plants.

Role of GAs in Floral Induction by Long Days

Not only is upregulation of the *LFY* promoter delayed in long-day-grown *ga1-3* plants, but the initial levels are also significantly reduced, suggesting that GAs play a role both in controlling the basal level of *LFY* promoter activity and in facilitating upregulation at a later time point. The first effect might be related to an essential function of GAs in flowering, regardless of environmental conditions, whereas the second effect might reflect an additional, partial requirement of GAs in the long-day-dependent pathway. To determine whether GAs are indeed required for the long-day-dependent upregulation of the *LFY* promoter, we grew *ga1-3 LFY*::*GUS* plants for 3 weeks in short days and then transferred them to long-day conditions. At this age, short-day-grown wild-

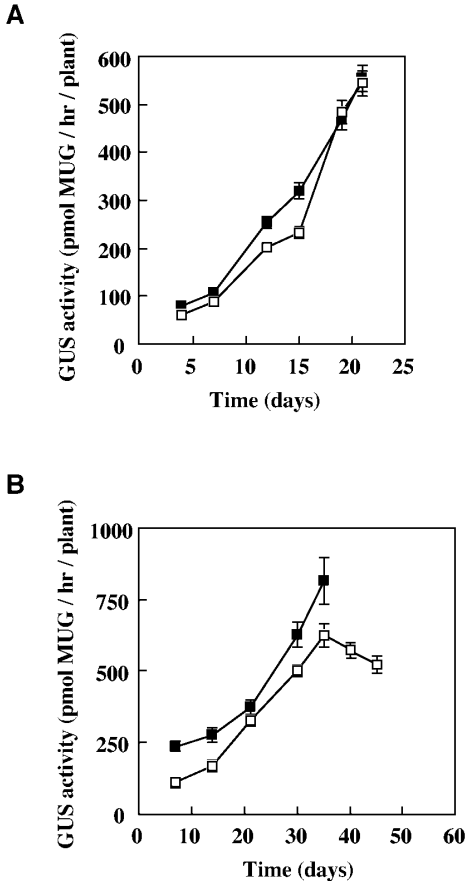

Figure 3. *LFY*::*GUS* Expression during Vegetative Growth of *spy-5* Mutants.

(A) LFY::GUS activity in long days. Plants homozygous for the *LFY*::*GUS* transgene in either a SPY^+ (DW150-304; open squares) or *spy-5* (304Y5; closed squares) background were grown in long days until flower buds were visible to the naked eye.

(B) LFY::GUS activity in short days. Symbols are the same as given in **(A)**.

Values are expressed as mean $\pm 2 \times$ SE of the mean ($n \geq 10$). Time represents days after sowing. Error bars that are not visible are smaller than the graph symbol. MUG, 4-methylumbelliferyl β-D-glucuronide.

type plants are competent to produce flowers immediately upon transfer to long days. If GAs were not involved in the long-day-dependent pathway, *LFY*::*GUS* upregulation and flower initiation upon transfer to long days should be similar between the *ga1-3* mutant and the wild type. In contrast to this scenario, we found that *LFY*::*GUS* induction in the *ga1-3* mutants increased much less rapidly than in control wild-type plants and that this defect correlated with a slower flowering response in *ga1-3* mutants (Figure 4).

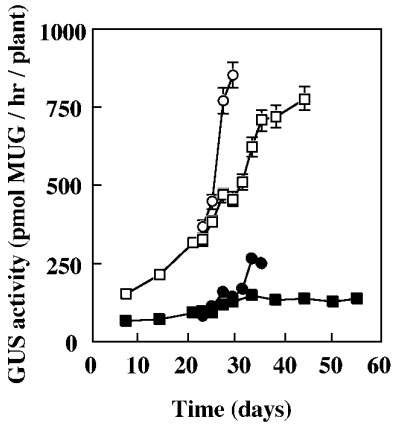

Figure 4. Induction of *LFY*::*GUS* Expression upon Transfer from Short to Long Days.

Plants homozygous for the *LFY*::*GUS* transgene in a *GA1*$^+$ (DW150-307; open symbols) or *ga1-3* (307G1; closed symbols) background were grown in short days for 21 days and either transferred to long days (circles) or left in short days as a control (squares). Values are expressed as mean $\pm 2 \times$ SE of the mean ($n \geq 10$). Time represents days after sowing. Error bars that are not visible are smaller than the graph symbol. MUG, 4-methylumbelliferyl β-D-glucuronide.

Suppression of Failure to Flower in *ga1* Mutants by Constitutive Expression of *LFY*

The failure to induce *LFY* promoter activity in short-day-grown *ga1-3* mutants suggests that the inability of *ga1-3* mutants to flower under noninductive conditions (Figure 5A) is at least in part caused by the absence of LFY activity. To determine how much of the *ga1-3* flowering defect can be attributed to the failure in *LFY* activation, we introduced a constitutively expressed version of *LFY*, in which *LFY* is under the control of the cauliflower mosaic virus 35S promoter (Weigel and Nilsson, 1995), into *ga1-3* mutants.

We found that the 35S::*LFY* transgene could restore the ability to flower to *ga1-3* mutants in short days (Figure 5B), although *ga1-3* 35S::*LFY* plants produced 28.0 ± 6.5 leaves compared with 15.8 ± 2.1 leaves for *GA1*$^+$ 35S::*LFY* plants (mean ±SD; $n = 15$). Thus, although *ga1-3* 35S::*LFY* could flower in short days, *ga1-3* still had an effect on flowering time in a 35S::*LFY* background, indicating that GAs regulate both *LFY* promoter activity and the competence to respond to *LFY* activity. An effect of *ga1-3* on the competence to respond to *LFY* activity was also observed in long days, in which *ga1-3* 35S::*LFY* plants produced 15.1 ± 1.4 leaves ($n = 15$) compared with 10.2 ± 1.0 leaves in *GA1*$^+$ 35S::*LFY* plants ($n = 32$).

A Rapid in Vitro Assay for Substances That Induce Flowering

Classic grafting experiments, such as those performed by Zeevaart (1958) four decades ago, have shown that the induction of flowering involves transmissible signals. Unfortunately, attempts to identify the biochemical nature of floral inductive substances have been largely unsuccessful. One reason for this might be the stringent criterion for the activity of such substances, that is, their ability to induce macroscopically visible flower primordia. Upregulation of the *LFY* promoter might present a more flexible standard with which to perform preliminary screens for candidate substances that induce flowering. Our analyses of *LFY* promoter activity in wild-type plants and *ga1-3* mutants under various environmental conditions suggest strongly that early events modulating *LFY* expression during the vegetative phase are physiologically relevant, because in all cases in which *LFY* promoter activity was upregulated during the vegetative phase, plants eventually flowered. Thus, in a screen for candidate substances that affect flowering, *LFY* promoter activity in young vegetatively growing plants could be a useful indicator.

To screen for such candidate substances in a high-throughput fashion, it is desirable to have an in vitro assay with which *LFY* promoter activity can be determined in a rapid and reliable manner. To adopt LFY::GUS activity measurements for screening purposes, we grew *LFY*::*GUS* seedlings in 96-well microtiter plates. Each well was filled with liquid MS medium, and each well held five seeds. After germinating seedlings had been incubated for 5 to 7 days in continuous light, they were challenged with various additions to the medium. GUS activity was measured after incubation for 3 additional days. Because of the previously established effects of GAs on LFY::GUS activity, we began by treating seedlings with GA_3, sucrose, or both. As shown in Figure 6, sucrose enhanced the vegetative expression of *LFY*::*GUS*, and this increase was potentiated by simultaneous incubation with GA_3, although incubation with GA_3 alone did not have a noticeable effect on *LFY*::*GUS* expression under these conditions.

As a control, we used a transgenic line carrying a fusion of the *GUS* gene to the promoter of a gene encoding a ubiquitin-conjugating enzyme. In young plants of this line, designated *AtUBC4*::*GUS*, GUS activity is also expressed at the shoot apex, thus mirroring *LFY*::*GUS* expression (Thoma et al., 1996). If the induction of *LFY*::*GUS* by sucrose and GA_3 was due simply to unspecific changes in growth rate, we

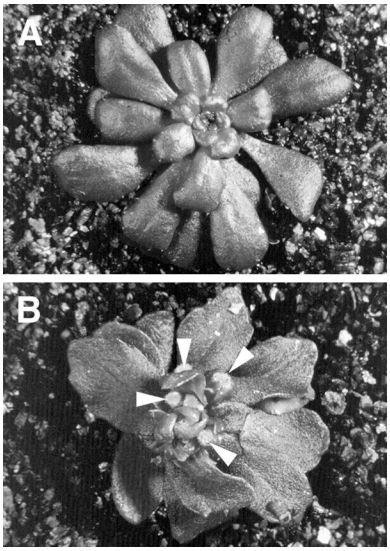

Figure 5. Rescue of the *ga1-3* Flowering Defect in Short Days by Constitutive Expression of *LFY*.

(A) Two-month-old *ga1-3* plant.
(B) Two-month-old *ga1-3* 35S::*LFY* plant. Flowers are indicated by arrowheads.

should see a similar effect in the *AtUBC4*::*GUS* line. Because none of the treatments affected AtUBC4::GUS activity, we conclude that the observed changes in LFY::GUS activity were specific (Figure 6).

Abscisic acid (ABA) counteracts GA activity in other situations, such as during seed development (Finkelstein and Zeevaart, 1994). To test further the validity of the in vitro assay, we treated seedlings with 0, 1, or 30 μM ABA, both in the absence of GA_3 and in the presence of increasing concentrations of GA_3. Incubation with ABA for 3 days did not have any obviously deleterious effect on seedling morphology. ABA treatment reduced *LFY*::*GUS* expression, regardless of the GA_3 concentration, including in the absence of exogenous GA_3. However, despite its negative effects on LFY::GUS activity, even the highest concentration of ABA did not eliminate LFY::GUS activity (Figure 7). The induction of LFY::GUS without exogenous ABA was saturated in the presence of 0.3 μM GA_3, whereas induction in the presence of either 1 or 30 μM ABA was saturated in 0.1 μM GA_3. That increasing GA_3 concentrations (up to 100 μM) could not overcome the inhibition by ABA indicates that GAs and ABA do not affect flowering in a competitive manner.

DISCUSSION

Flower initiation is under the control of both environmental and endogenous signals. Because the specification of individual flowers is controlled by floral meristem identity genes, their activity somehow must be controlled by floral-inductive signals. The study of Arabidopsis mutants defective in GA biosynthesis or signaling has demonstrated that endogenous GAs are involved in the promotion of flowering, although the requirement for GAs in short days is more critical (Wilson et al., 1992; Jacobsen and Olszewski, 1993). Previous studies addressing the link between the genetic activity of floral meristem identity genes and GAs have emphasized events that occur after the transition to flowering has been made (Okamuro et al., 1996, 1997). Here, we have focused on how GAs control when the transition to flowering is made, and how these effects correlate with activation of the promoter of the floral meristem identity gene *LFY*.

Role of GAs in the Flowering of Arabidopsis

GA-dependent signaling has been suggested to be part of a pathway that promotes flowering in the absence of external floral-inductive stimuli (Martínez-Zapater et al., 1994). We have demonstrated that one of the targets of this pathway is the *LFY* promoter (Blázquez et al., 1997; this study). The significance of our observation lies in the fact that *LFY* expression and flower formation are not strictly coupled, because *LFY* expression in the wild type precedes flower formation

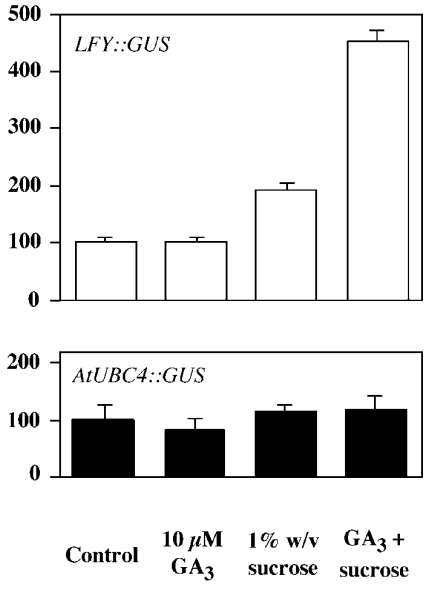

Figure 6. Effect of Sucrose and GA_3 on *LFY*::*GUS* Expression in Seedlings Grown in Vitro.

The ordinates indicate relative GUS activities in arbitrary units. The values represent the average of four replicate experiments; the error bars indicate one standard error of the mean.
(Top) LFY::GUS.
(Bottom) AtUBC4::GUS control.

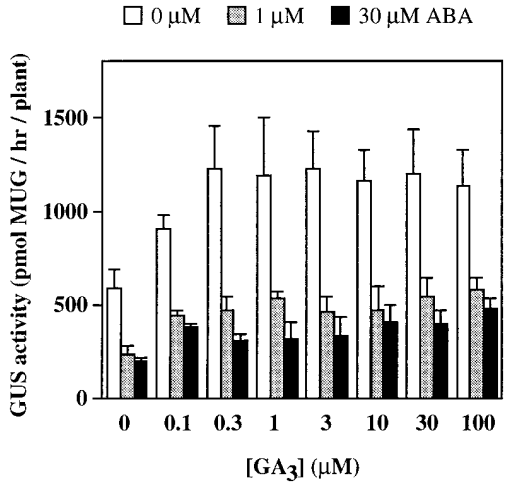

Figure 7. Effect of ABA on *LFY*::*GUS* Expression in Seedlings Grown in Vitro.

The experiments were performed in the presence of 1% [w/v] sucrose. The values represent the average of four replicate experiments; the error bars indicate one standard error of the mean. MUG, 4-methylumbelliferyl β-D-glucuronide.

(Blázquez et al., 1997; Hempel et al., 1997). However, upregulation of the *LFY* promoter appears to be an indicator of subsequent flowering, because failure to flower in short days in the GA-deficient *ga1-3* mutant goes hand in hand with the elimination of *LFY* upregulation.

That expression of *LFY* from a constitutive promoter can rescue the flowering defect of *ga1-3* mutants is consistent with the idea that the failure of *ga1-3* mutants to flower in short days is caused by the failure to upregulate *LFY.* However, GAs apparently play a role not only in the activation of the *LFY* promoter but also in controlling competence to respond to *LFY* activity, because 35S::*LFY ga1-3* plants produced more leaves than did 35S::*LFY GA1*$^+$ plants.

We have also tried to test the hypothesis that GAs mediate the effects of a long-day-independent, autonomous flowering pathway by examining flowering and *LFY* promoter activity in *ga1-3* plants grown in complete darkness. Surprisingly, the requirement of GAs for flowering is less critical in darkness than in short days (Roldán et al., 1997). Unfortunately, the conclusions that can be drawn from these observations are hindered by several characteristics of growth in the dark. First, because the morphology of dark-grown plants is so different from light-grown plants, it is impossible to determine whether leaf number (or chronological time) can be taken as a directly comparable indicator of flowering time in a developmental sense. Second, although most photoreceptors, such as phytochrome A, promote flowering, others, such as phytochrome B, inhibit flowering (Goto et al., 1991; Reed et al., 1993, 1994; Whitelam et al., 1993). Therefore, it is difficult to predict the result of all photoreceptors being inactive, which should be the case in complete darkness. Third, to overcome the developmental arrest of etiolated seedlings, it is necessary to bring the shoot into contact with medium containing sucrose, which itself has been proposed to be a constituent of the flowering signal (Bernier et al., 1993). Nevertheless, that flowering in darkness is severely delayed by the *ga1-3* mutation indicates that flowering in darkness relies to a large extent on GAs (Roldán et al., 1997).

Role of GAs under Inductive Photoperiods

Compared with short days, the flowering defect of *ga1-3* mutants is relatively minor in long days (Wilson et al., 1992; Silverstone et al., 1997). This observation could be rationalized by proposing that a long-day pathway involving the flowering-time gene *CO* parallels the GA-mediated pathway (Putterill et al., 1995). *LFY* expression is genetically a direct target for the long-day pathway, because forced expression of *CO* in a transgenic system triggers a rapid increase in *LFY* expression (Simon et al., 1996). Additional evidence for the redundancy between the *CO* and GA pathways comes from the observation that treatment of *co* mutants with paclobutrazol prevents the upregulation of the *LFY* promoter in long days (M.A. Blázquez and D. Weigel, unpublished results).

However, simple redundancy does not fit with the delayed and weak upregulation of the *LFY* promoter upon the shift from short to long days. We interpret this discrepancy as being an indication of GA involvement in the long-day-dependent, facultative pathway promoting flowering. A separate piece of circumstantial evidence for an interaction between long days and GA signaling is that long days cause increased expression of GA biosynthetic genes, such as *GA5*, which encodes a GA 20-oxidase (Xu et al., 1997).

Another surprising observation has been the severe reduction of *LFY* promoter activity in *ga1-3* mutants during the first 2 weeks of growth in long days. This effect is specific for *ga1-3* mutants. It has not been observed in several other late-flowering mutants, although most of these mutants flower later in long days than do *ga1-3* mutants (O. Nilsson, I. Lee, M.A. Blázquez, and D. Weigel, manuscript in preparation). A similar differential effect on vegetative phase change has been observed in *ga1-3* and other late-flowering mutants. The production of abaxial trichomes on leaves and bracts, an indicator of vegetative phase change, is blocked by the *ga1-3* mutation in both long and short days (Chien and Sussex, 1996; Telfer et al., 1997). Similar to the observations for *LFY* promoter activity, none of the late-flowering mutations examined by Telfer et al. (1997) abolished abaxial trichome production, although several flowered later than did *ga1-3* mutants in long days. Thus, in *ga1-3* mutants grown in long days, vegetative phase change and the transition from vegetative to reproductive growth are partially uncoupled. The scenario emerging from these observations is that *ga1-3* mutants never reach the same level of reproductive

competence as wild-type plants do and that whereas long days can ultimately induce flowering and *LFY* promoter activity in *ga1-3* mutants, they do so more inefficiently in *ga1-3* mutants than in wild-type plants.

One observation that still needs explanation is the apparent paradox in the flowering behavior of *ga1-3* and other mutants that flower late in both long and short days. In short days, the *ga1-3* mutant shows the most extreme phenotype and is the only one that never flowers, indicating that the long-day-independent pathway is more strongly affected in the *ga1-3* mutant than in any other late-flowering mutant. In contrast, several late-flowering mutants that are delayed in both short and long days, such as *fca* and *fve*, flower later than do *ga1-3* mutants in long days (Koornneef et al., 1991; Wilson et al., 1992). From these observations, one cannot conclude whether GAs and flowering-time genes, such as *FCA* and *FVE*, act in the same pathway. However, it is possible to investigate the functional relationships between these factors by determining the effect of these late-flowering mutations on flowering and *LFY* promoter activity in a situation in which GA biosynthesis is inhibited in long days.

Mechanistic Links between GAs and *LFY* Expression

The specific tissues in which GAs are required to promote flowering in Arabidopsis are not known, and little is known in general about the molecules that transduce the effects of GA. The only protein that has been directly implicated in the transcriptional control of downstream target genes is the barley transcription factor GAMyb, whose RNA accumulation is under the control of GAs and which positively regulates an α-amylase promoter in aleurone (Gubler et al., 1995). Although the *LFY* promoter contains several sequences that resemble the Myb-binding motif, thus far we have no evidence that these motifs are required for *LFY* promoter activity. However, with the system established here, we can continue to dissect the link between *LFY* promoter activity and GAs and compare the mechanisms of GA action in flowering with those in other developmental processes.

METHODS

Plant Material

Two *Arabidopsis thaliana* Landsberg *erecta* *LFY*::*GUS* lines (DW150-304 and DW150-307) and one Nossen line (DW150-12), which is homozygous for a fusion between the 2.3-kb *LEAFY* (*LFY*) promoter and the *uidA* gene encoding β-glucuronidase (GUS), have been previously described (Blázquez et al., 1997; Hempel et al., 1997). The *AtUBC4*::*GUS* line in ecotype RLD has been described by Thoma et al. (1996). DW151.2.5 contains the 35S::*LFY* transgene (Weigel and Nilsson, 1995) in the Landsberg *erecta* background. The mutant line *ga1-3*, which is in the Landsberg *erecta* background, has been described previously (Koornneef and Van der Veen, 1980; Koornneef et al., 1985). *spy-5* is a mild *spindly* allele in the Landsberg *erecta* background and was a kind gift of N. Olszewski (University of Minnesota, St. Paul).

Line 307G1 was constructed by crossing DW150-307 to *ga1-3* plants. Transgenic plants homozygous for *ga1-3* were initially identified by their short stature and dark green color and confirmed by polymerase chain reaction (Silverstone et al., 1997). Lines that were homozygous for the transgene were identified by testing F_3 progeny. Line 304Y5 was derived from a cross between DW150-304 and a *spy-5* plant. From the F_2 generation, plants homozygous for the *spy-5* allele were identified by their ability to germinate on Murashige and Skoog (MS; Murashige and Skoog, 1962) plates with 40 μM paclobutrazol (Zeneca Ag Products, Wilmington, DE) and by their slender phenotype, light green color, and poor seed yield. Plants homozygous for the transgene were selected from F_3 progeny. *ga1-3* 35S::*LFY* plants were constructed by backcrossing DW151.2.5 plants twice to *ga1-3* mutants.

Growth Conditions

Before sowing, seeds were stratified for 2 to 3 days at 4°C. Plants were grown at 23°C in long (16 hr of light and 8 hr of dark) or short (9 hr of light and 15 hr of dark) days under a mixture of 3:1 cool-white and Gro-Lux fluorescent lights (Osram Sylvania, Danvers, MA). To grow plants in the dark, seeds were kept after stratification for 4 to 6 hr under white light to induce germination, and they were then sown on MS plates with 1.5% sucrose. Plates were wrapped in two layers of aluminum foil and incubated vertically.

ga1-3 mutants require exogenous gibberellins (GAs) to germinate and were incubated with 50 μM GA_3 (Sigma) during stratification. Seeds were rinsed thoroughly with water before sowing. Application of exogenous GA_3 during vegetative growth was achieved by spraying soil-grown plants twice weekly with a solution of 100 μM GA_3 and 0.02% Tween-20 (Bio-Rad).

GUS Staining and Activity Measurements

For histochemical analyses and quantitative measurements of GUS activity, samples of plants grown on soil or MS plates were collected and treated as previously described (Blázquez et al., 1997).

For the in vitro assays, seeds were sterilized for 5 min in 70% ethanol containing 1% Triton X-100 (Sigma) at room temperature. Seeds were rinsed twice with 95% ethanol and air dried in a laminar flow hood. Five seeds were placed in each well of a 96-well microtiter plate containing 100 μL of MS medium containing 1.5 g/L Mes and adjusted to pH 5.7 with KOH. Germinating seedlings were incubated on a rotating shaker at 30°C under fluorescent lights. After 5 to 7 days, seedlings were challenged by various additions and returned to the rotating shaker for an additional 2 days. The in vivo GUS assay was initiated by replacing the growth medium with 100 μL of the following solution: 50 mM sodium phosphate, pH 7, 10 mM mercaptoethanol, 10 mM EDTA (disodium salt), 0.1% [w/v] SDS, 0.1% [w/v] Triton X-100, 2% isopropanol, and 440 mg/L 4-methylumbelliferyl β-D-glucuronide. After incubation for 1 to 4 hr at 37°C with slow circular agitation, the reaction was stopped by adding 100 μL of 0.2 M Na_2CO_3, and fluorescence intensity was determined on a microtiter plate reader, which excites and reads from above, with an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

RNA Analysis

Total RNA was extracted from the apices of 4-week-old plants by using the RNeasy Plant Mini Kit (Qiagen, Santa Clarita, CA). Reverse transcription coupled to polymerase chain reaction (RT-PCR) was conducted with the Titan One Tube RT-PCR System kit (Boehringer Mannheim) by using 1 μg of total RNA. A *LFY* fragment was amplified using oligonucleotides 5′-TGAAGGACGAGGAGCTT-3′ and 5′-TTGCCACGTGCCACTTC-3′ as primers. As a control, a fragment from the gene encoding eukaryotic protein synthesis initiation factor 4A (Metz et al., 1992) was amplified with two oligonucleotides, namely, 5′-TTCTCAAACCATAAGCATAAATACCC-3′ and 5′-AAACTCAATGAAGTACTTGAGGGACAAG-3′. Aliquots were removed from the reaction mixture after 8, 10, and 12 cycles of PCR, separated on an agarose gel, and transferred onto a membrane. A DNA gel blot was hybridized with radiolabeled *LFY* and *eIF4A* cDNA probes. Signal intensities were determined with a Molecular Dynamics (Sunnyvale, CA) PhosphorImager, and values from the exponential range of amplification were compared.

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