The Plant Journal (2001) **25**(5), 509–519

Gibberellin response mutants identified by luciferase imaging

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Received 25 September 2000; revised 8 December 2000; accepted 11 December 2000.
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Summary

The gibberellin (GA) 20-oxidase encoded by *Arabidopsis GA5* catalyzes the synthesis of active GAs. *GA5* is a regulatory step in GA biosynthesis as *GA5* mRNA levels are negatively regulated by its bioactive GA products. A fusion between the *GA5* promoter and the firefly luciferase reporter (*GA5-LUC*) was shown to be similarly regulated, indicating GA feedback of *GA5* occurs at the transcriptional level. The fidelity of the *GA5-LUC* reporter permitted a fusion genetic screen to identify mutants altered in transgene expression. This bioimaging screen identified two types of recessive mutants with increased LUC activity and apparent GA-related growth phenotypes, a dwarf (*lue1*) and two late flowering mutants (*fpa1-3* and *fpa1-4*). Mutant progeny exhibited altered levels of LUC and of endogenous *GA5* and other GA-regulated mRNAs. SSLP-based mapping localized *lue1* to chromosome I near the *ga2* locus, although complementation analyzes showed that *lue1* is not allelic to *ga2*. Mapping and complementation analyzes showed that the late flowering mutants are allelic to *fpa1*. This provides genetic evidence for crosstalk between the autonomous and gibberellin-dependent flowering pathways.

Keywords: dwarf, *GA5*, GA 20-oxidase, late flowering, luciferase.

Introduction

The diterpenoid gibberellins influence physiological and developmental processes in plants including seed germination, stem elongation and flower and fruit development (Hooley, 1994; Silverstone *et al.*, 1997). Genetic approaches have identified mutants exhibiting altered germination and growth phenotypes in the presence or absence of exogenous GA or GA biosynthetic inhibitors (Koornneef *et al.*, 1985). Molecular cloning of mutant loci have identified GA biosynthetic and signaling components. One class of such mutants exhibits GA-insensitivity and includes *D8–1* from maize, *Rht3* from wheat and *gai/rga* from Arabidopsis (Hooley, 1994; Koornneef *et al.*, 1985; Peng *et al.*, 1997; Silverstone *et al.*, 1998). Another class exhibits constitutive GA responses and includes *la* and *crys* from pea, *pro* from tomato, *sln* from barley and *spy* from *Arabidopsis thaliana* (Hooley, 1994; Jacobsen and Olszewski, 1993). Current studies of these genes provide insight into the complexity and agronomic importance of GA-mediated signaling pathways (Peng *et al.*, 1999a). For example, *GAI*, *RGA* and a third related gene may act in a pathway together with *SPY* to negatively regulate GA responses (Silverstone *et al.*, 1998). GA-mediated responses through them include both the induction of downstream genes such as *GASA1* (Herzog *et al.*, 1995; Phillips and Huttly, 1994), as well as the repression of genes involved in the synthesis of active GAs from inactive precursors (Silverstone *et al.*, 1998).

Genes encoding the major enzymes of GA biosynthesis and metabolism have also been identified (reviewed by Lange, 1998). These include *Arabidopsis GA1* (*ent*-copalyl pyrophosphate synthase; Sun and Kamiya, 1994), *GA2* (*ent*-kaurene synthase: Sun and Kamiya, 1994; Yamaguchi *et al.*, 1998a), *GA3* (*ent*-kaurene oxidase; Helliwell *et al.*, 1999), *GA4* (3β-hydroxylase; Chiang *et al.*, 1995), *GA5* (GA 20-oxidase; Phillips *et al.*, 1995; Xu *et al.*, 1995) and, more recently, GA 2-oxidases involved in the inactivation of GAs (Thomas *et al.*, 1999). The synthesis of active GAs is

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negatively feedback regulated via GAI, RGA and SPY which directly or indirectly affect the expression of GA 20-oxidase (Phillips *et al.*, 1995; Xu *et al.*, 1995) and 3β-hydroxylase (Chiang *et al.*, 1995; Yamaguchi *et al.*, 1998b). For example, *gai* mutants accumulate heightened levels of both active GAs and of *GA5* 20-oxidase mRNA (Peng *et al.*, 1999b).

As an alternative to earlier phenotypic screens, we have described a fusion genetic approach to identify *trans*-acting mutations affecting the expression of a transgene composed of the GA-responsive *GASA1* promoter fused to the firefly luciferase reporter (*LUC*; Raventos *et al.*, 2000). We present here a similar strategy with the *GA5* GA 20-oxidase promoter fused to *LUC*. The *GA5* promoter was chosen because it is negatively feedback-regulated by active GAs such that the *cis*-acting element(s) in the promoter are likely target(s) for GA signaling pathways. The LUC reporter was used because its activity can be easily measured in a non-destructive, *in vivo* assay (Kost *et al.*, 1995) but is sufficiently unstable to serve as a marker for transcription from different promoters (Millar *et al.*, 1992). We report the results of this screening and analyses of mutants exhibiting altered levels of LUC activities associated with GA-related phenotypes.

Results

Production and initial analysis of transgenic plants

5′ upstream sequences the *GA5* 20-oxidase gene were isolated from an Arabidopsis (ecotype Col0) genomic library by long-range PCR. The *GA5* gene sequence, including promoter sequences used here, subsequently became available (GenBank accession number AL079350, complement from nt41778). The *GA5* promoter was used to generate transcriptional fusions with the *E. coli GUS* and firefly *LUC* reporters and constructs carrying either 900 bp or 500 bp of promoter were stably transferred to plants via Agrobacterium-mediated transformation.

Twenty-five lines expressing GUS were initially examined for expression patterns from the *GA5*-500-*GUS* and *GA5*-900-*GUS* constructs. Similar GUS histochemical stainings were found in all lines. Five-day-old seedlings exhibited strong GUS activity in the transition region between hypocotyl and root, and in cells at the rim of cotyledons (Figure 1a). At the rosette stage, staining was observed in the leaf petiole base, in cells of the leaf rim, in the hydathodes and in the abaxial leaf midrib (Figure 1b). This pattern of staining is similar to that seen for the Arabidopsis *GA1* encoding the *ent*-kaurene synthase A (Silverstone *et al.*, 1997). Expression was also pronounced in epidermal cells of the trichome base (Figure 1c), and in stomatal guard cells (Figure 1d). This result is consistent with the role of GAs in trichome development through *GLABROUS1* (*GL1*) regulation in Arabidopsis (Perazza *et al.*, 1998).

GA feedback regulation of GA5-LUC reporters

Seedlings of the WT transgenic carrying either the 900 bp or the 500 bp fragment of the *GA5* promoter fused to LUC were then assessed for LUC reporter expression *in vivo*.

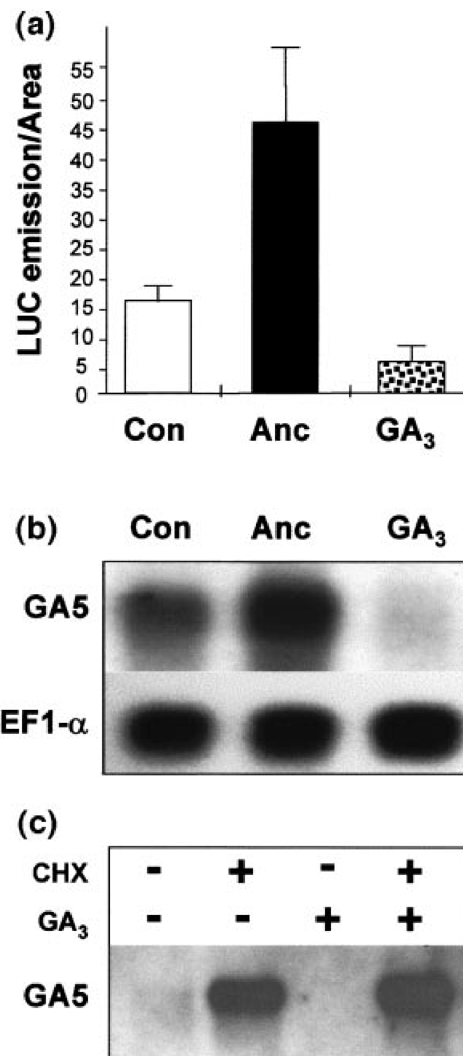


Figure 2. *GA5* regulation by GA or the GA biosynthetic inhibitor ancymidol.
(a,b) Seedlings grown for 5 days in liquid MS followed by 24 h treatment with 50 μM GA_3 or 4 μM ancymidol. (a) Relative *in vivo* LUC activities acquired by CCD camera. (b) Northern analysis of *GA5* mRNA accumulation. 1 μg Poly A^+ RNA per lane with the EF1-α riboprobe as loading control.
(c) Northern analysis of *GA5* mRNA accumulation following treatment with translational inhibitors (CHX). Five-day-old-seedlings were transferred to 50 ml liquid 0.5 × MS in flasks for 1 day prior to treatment. The translational inhibitors cycloheximide (50 μM) and chloramphenicol (50 μM) were added to the medium 2 h before GA treatment (50 μM GA_3 for 16 h).

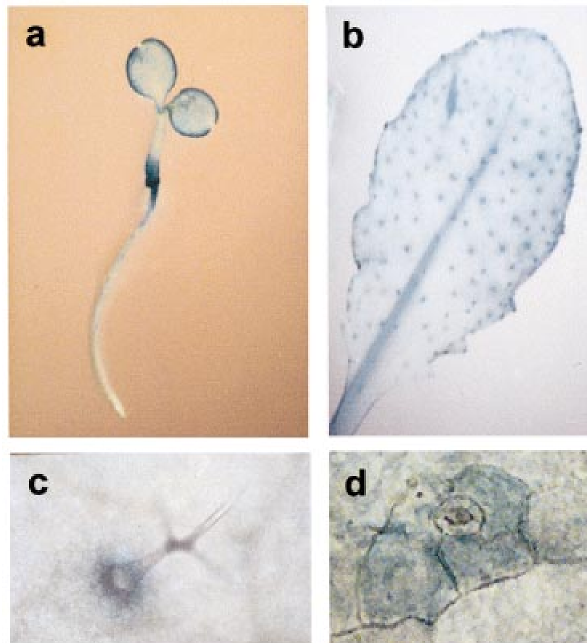
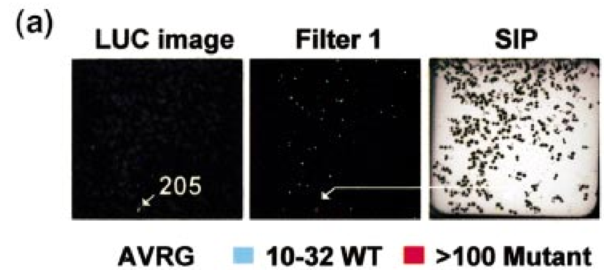


Figure 1. Histochemical localization of GUS activity from the *GA5-500-GUS* reporter.
GUS expression in (a) 5-day-old seedlings germinated on MS without GA; (b) leaf of a 3-week-old plant; (c) basal cells of a trichome; (d) guard and companion cells.

LUC activity was monitored in 5-day-old seedlings grown on MS plates supplemented with 50 μM GA_3 or 4 μM of the GA biosynthetic inhibitor ancymidol (Rademacher, 2000). Upon GA_3 treatment, the LUC activity of both promoter constructs was reduced by 50% compared to the control treatment, whereas ancymidol treatment resulted in a twofold induction (Figure 2a). This indicated that the *cis*-element(s) responsible for negative GA feedback regulation is located within 500 bp upstream of the transcription start site of *GA5*. As a control for GA and ancymidol treatments, poly A^+ RNA was extracted to assess regulation of the endogenous *GA5* GA 20-oxidase by Northern hybridization. This indicated that expression of the *GA5-LUC* reporter mimicked that of endogenous *GA5* (Figure 2b). Furthermore, F_2 progeny from a cross between the WT transgenic and the GA biosynthetic mutant *ga1-1* showed enhanced LUC activity in *ga1-1* seedlings (data not shown). These results confirm that *GA5* is negatively regulated by GA, as previously observed in the GA-deficient *ga1-2* mutant (Phillips *et al.*, 1995; Xu *et al.*, 1995), and that this regulation occurs at least in part at the transcriptional level.

To investigate whether *de novo* protein synthesis is required for *GA5* feedback regulation, plantlets were treated with GA_3 and the translational inhibitors cycloheximide and chloramphenicol. Northern hybridization on poly-A^+ RNA revealed a dramatic increase in *GA5* mRNAs levels following treatment with the translational inhibitors



(b)

LINE	Fold induction CON	GA$_3$	ANC
WT	1.0	0.6	1.8
loe1	0.3	0.1	0.5
loe2	1.0	0.4	1.0
loe3	0.2	0.1	0.1
lue1	2.0	2.1	3.5
lue2	1.9	1.1	7.0
lue3	1.0	0.9	7.5
lue4	2.2	1.6	12.0
lue5	2.1	2.3	7.9
lue6	2.0	1.5	5.6
lue7	1.4	1.4	5.8
fpa1-3	20.0	14.0	57.0
fpa1-4	18.0	11.0	45.0

(c)

	Seedlings Tested	F2 Phenotype WT	Mutant	Seg.	χ^2
lue1	416	332	84	3.9 :1	0.06
fpa1-3	397	303	94	3.2 :1	0.001
fpa1-4	495	379	116	3.2 :1	0.008

Figure 3. *In vivo* LUC screen and activities of WT and selected mutants in response to GA_3 and ancymidol treatments.
(a) Bioluminescence image of some 200, 5-day-old seedlings recorded after background subtraction of two images (LUC image, left panel). The LUC image was filtered to visualize high LUC expressors (red, average grey values (AVRG) > 100 (U), among average expressors (blue, AVRG between 10 and 32 U; center panel). An arrow designates an *fpa1-3* seedling (AVRG = 205 U). Superposition of images identifies seedlings for transfer to soil (SIP, right panel).
(b) Relative *in vitro* LUC activities in *L*UC *l*ow *e*xpressor (*loe*) and *L*UC s*u*per *e*xpressor (*lue*) mutants. Selected mutants were selfed and some 100 progeny grown on MS with kanamycin without or with 50 μM GA_3 or 4 μM ancymidol. The data are presented as fold expression compared to non-treated WT (con = 1.0).
(c) Genetic characterization of *lue1* and *fpa1-3 and fpa1-4*. Mutants were backcrossed to WT transgenic and F_2 progenies scored for phenotype and LUC activity. (seg.) segregation ratio.

with or without GA_3 (Figure 2c). A similar result was observed in treatments of the *ga5* mutant (data not shown). This indicates that *GA5* mRNA levels are regulated by protein factor(s) which may include a labile transcrip-

tional repressor and/or a factor modulating *GA5* mRNA stability.

Screening of mutants with altered GA5-LUC expression

100 000 M_2 seedlings from bulked, γ-irradiated seeds of the WT transgenic parent expressing *GA5-LUC* containing 500 bp of the *GA5* promoter were imaged for LUC activity after 5 days of growth on MS plates supplemented with 50 μM GA_3. This initial screen allowed the isolation of about 40 LUC-super-expressors (*lue*) and 40 LUC-low-expressors (*loe*). As an example, the images leading to the isolation of the very high LUC expressing *fpa1-3* mutant are presented in Figure 3(a). This shows that super-imposition on the bioluminescent image (left) of color code filters 1 and 2 (middle) permitted the selection of this seedling from the light reference images in which individual seedlings are visible (right). Following imaging, selected M_2 plants transferred to soil were allowed to self and altered LUC expression patterns re-examined in progeny. The second LUC screen of progeny confirmed inappropriate LUC expression in only 12 lines derived from separately mutagenized pools. The relative LUC activities of these lines was then assayed quantitatively in extracts of seedlings treated without or with GA_3 or ancymidol (Figure 3b).

Initial genetic analyses

Three of the mutants exhibited visible phenotypes, one a dwarf (*lue1*) and two late flowering plants (*fpa1-3, fpa1-4*). Because these growth phenotypes may be related to alterations in GA biosynthesis or sensitivity, genetic characterization of the three mutants was initiated by back-crossing to the transgenic WT. Phenotypes and LUC activities of all F_1 plants were similar to the WT transgenic parent, indicating that the three mutations were recessive. F_2 segregation analyses revealed that the mutations, scored both for high LUC activities and phenotypic characters, segregated as monogenic traits (Figure 3c).

Characterization of lue1

When grown under long days (LD) for 4 weeks, *lue1* exhibited semi-dwarfism, reaching only 30% of WT height, and had reduced numbers of secondary shoots, short compressed leaves and flower parts (Figure 4a–d). *Lue1* siliques harbored about 20% the number of seeds as in WT, although backcrosses to WT with *lue1* as the pollen donor produced a normal seed set. This suggests that the lower seed set of *lue1* may be primarily due to reduced filament and stamen height in the mutant (Figure 4c,d). Interestingly, *lue1* seeds were rounder and slightly bigger than WT (Figure 4e,f), due to an abnormal cotyledon shape, which was clearly visible early in germination.

Northern blots with GA-related probes were performed on Poly A^+ RNA extracted from control or GA_3-treated *lue1* seedlings. Steady state levels of *GA5* mRNA were higher in *lue1* than WT (Figure 4g), as was also seen for *GA5-LUC* activity (Figure 3b). Interestingly, *lue1 GA5* mRNA levels increased following GA_3 treatment, unlike the WT parental which exhibited the expected *GA5* negative feedback regulation by GA (Figure 4g). A similar result was observed for *GA4* mRNA. This indicates that *lue1* is compromised in an aspect of a feedback loop regulating the expression of GA4 and GA5, both key enzymes in the conversion of inactive precursors to active GAs. In contrast, while *GAI* mRNA levels were reduced in *lue1* compared to WT, *GAI* mRNAs increased slightly upon GA treatment in *lue1* and in WT (not shown). This indicates that the normal, positive regulation of *GAI* by GA is not compromised in *lue1* (Peng *et al*., 1997) and that feedback control of GA-biosynthetic and GA-signaling genes may involve at least two independent components.

Genetic mapping revealed that *lue1* was linked to marker nga692 on the bottom of chromosome I (2 recombination events out of 37 plants examined), in the vicinity of *GA2* encoding ent-kaurene synthase (Yamaguchi *et al*., 1998a). The GA-deficient *ga2* mutant is the unique dwarf mutant characterized so far in this region of the chromosome. Crosses between *lue1* and *ga2-1* produced wild-type progeny, indicating that *lue1* is not allelic to *ga2-1*. In addition, *lue1* semi-dwarfism was not restored to wild-type growth by repeated applications of GA_3 (data not shown). These data suggest that *lue1* is not a GA-biosynthetic mutant, and represents a novel semi-dwarfing locus.

Phenotypes of fpa1-3 and fpa1-4

Fpa1-3 and *fpa1-4* originated from different mutagenized pools, and their similar phenotypes suggested that they might be allelic. Both mutants had markedly longer hypocotyls, cotyledons and first leaves than WT, although their subsequent growth rates and leaf numbers were similar until the WT bolted (not shown). Thereafter, *fpa1-3* and *fpa1-4* were delayed in flowering and exhibited enhanced vegetative growth compared to WT under long day (LD; Figure 5a). In contrast, *fpa1-3* and *fpa1-4* showed marked decreases in biomass and leaf size, though not leaf number, under SD compared to WT whose growth was enhanced (Figure 5d).

Measurements of flowering time (FT) for *fpa1-3*, *fpa1-4* and the WT transgenic grown under LD or SD are shown in Figure 5(b). Under LD, *fpa1-3* and *fpa1-4* flowered after 55/57 days while WT flowered in only 38 days. Under SD, *fpa1–4* flowered after 146 days while WT flowered in 53 days. Under SD, *fpa1-3* plants did not flower and

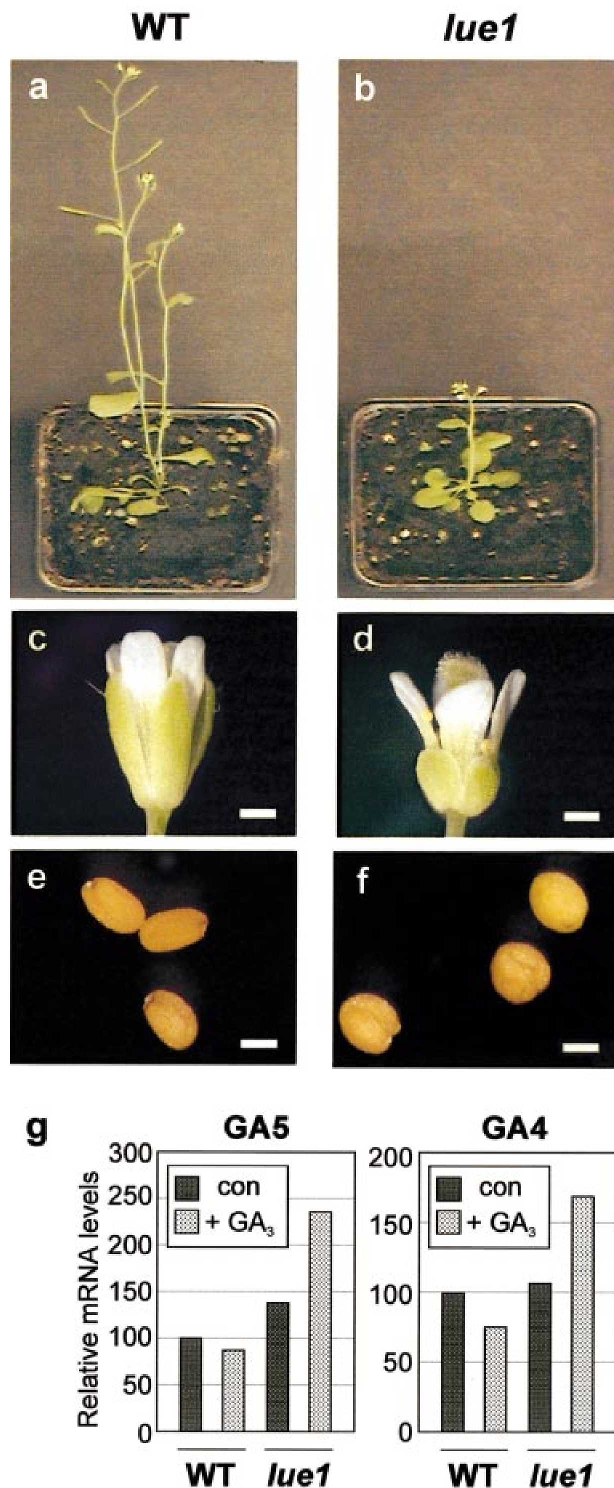


Figure 4. *Lue1* semi-dwarf with altered flower and seed development and GA responses.

(a, b) WT and *lue1* phenotypes at flowering under LD. (c, d) WT and *lue1* flowers showing reduced *lue1* floral parts. (e, f) WT and oblong *lue1* seeds. Bars represent 0.1 mm. (g) Northern analysis of *GA5* and *GA4* mRNA levels in WT and *lue1* upon GA_3 treatment. 1 µg poly A^+ RNA was loaded per lane, hybridized to riboprobes, and standardized (WT con = 100) to signals from subsequent hybridization with an EF1-α probe.

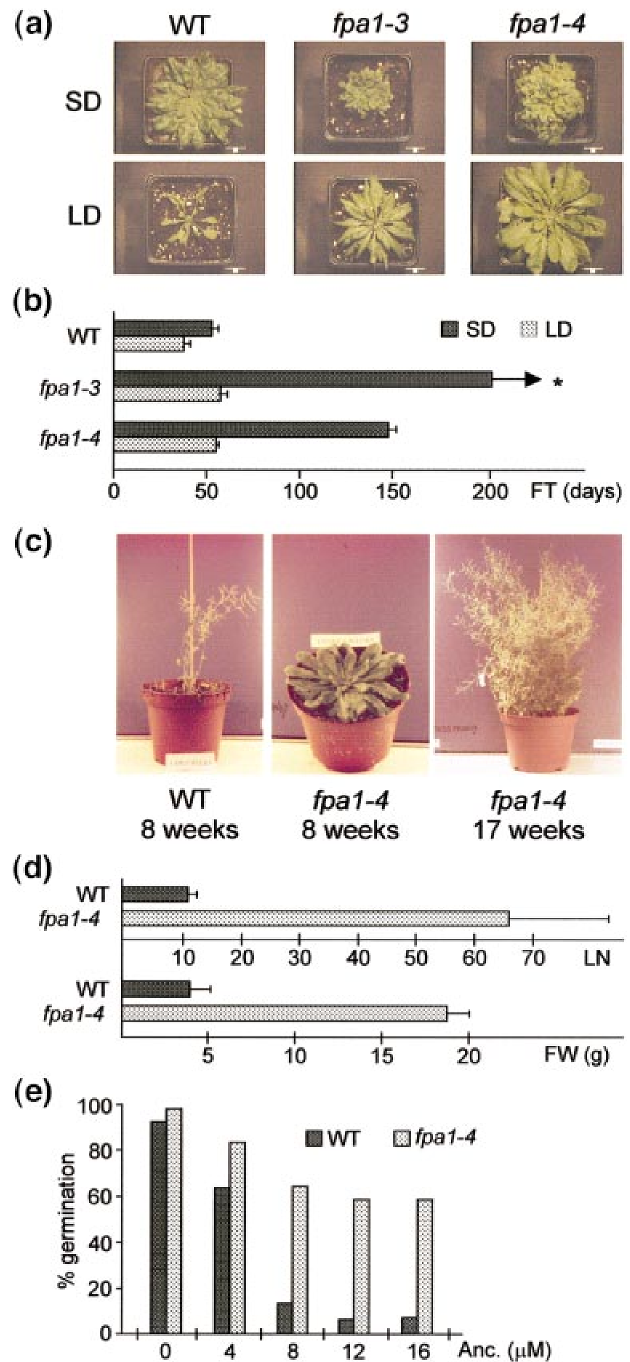


Figure 5. *Fpa1-3 and fpa1-4* exhibit late-flowering and reduced sensitivity to the GA biosynthesis inhibitor ancymidol.

(a) WT and *fpa1-3 and fpa1-4* phenotypes at 3 weeks under SD (10 h) or LD (20 h).

(b) WT and *fpa1-3 and fpa1-4* flowering time at first open flower (FT) under SD or LD.

(c) WT and *fpa1-4* at 8 weeks in LD (left and center). *Fpa1-4* at 17 weeks (right).

(d) WT and *fpa1-4* leaf numbers (LN) and vegetative mass (g fresh weight) at first open flower (LN).

(e) Percentage of WT and *fpa1-4* seed germination on increasing concentrations of ancymidol (Anc).

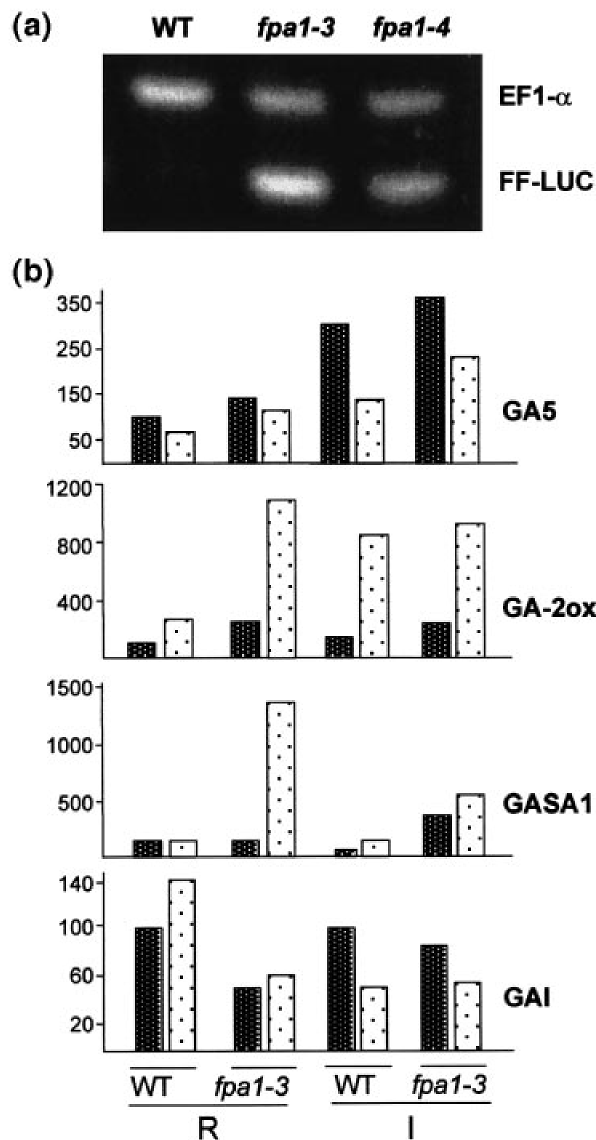


Figure 6. *Fpa1-3* and *fpa1-4* exhibit altered LUC activity and GA-related mRNA accumulation.
(a) RT-PCR quantitation of LUC mRNA in WT and *fpa1-3* and *fpa1-4*. RT-PCR was performed on 200 ng total RNA template. EF1-α cDNA was used as internal control and was coamplified with LUC cDNA.
(b) Northern analysis of *GA5, GA-2-oxidase* (GA-2ox), *GASA1* and *GAI* mRNAs in WT and *fpa1-3* in rosette leaves (R) and inflorescences (I) upon GA_3 treatment.

eventually wilted without producing bolts after 200 days. Since both *fpa1-3* and *fpa1-4* exhibit a late flowering phenotype associated with an increase in time to flowering under SD, they belong to the autonomous late flowering class of mutants (Fowler *et al.*, 1999; Koornneef *et al.*, 1998). As an example, *fpa1-4* was still in a vegetative stage after 8 weeks under LD conditions while the parental was setting seeds (Figure 5c). As might be expected (Koornneef *et al.*, 1991; Sheldon *et al.*, 1999), *fpa1-4* produced six times as many leaves and roughly five times the leaf biomass prior to flowering as did WT (Figure 5d). When grown under optimal glass house conditions, *fpa1-3* and *fpa1-4* also produced some six times as many seed as WT (data not shown).

To assess whether *fpa1-3* and *fpa1-4* were altered in their sensitivity to active GAs, GA_3 was repeatedly applied to the mutants and WT until bolting. Whereas WT exhibited both vegetative growth enhancement and bolting induction upon GA_3 treatment, *fpa1-3* and *fpa1-4* did not (data not shown). The sensitivity of *fpa1-4* to ancymidol, an inhibitor of the GA biosynthetic enzyme *ent*-kaurene synthase (Rademacher, 2000), was also examined. When WT and *fpa1-4* seeds were germinated on MS plates supplemented with increasing concentrations of ancymidol, *fpa1-4* was significantly less affected than WT (Figure 5e). At high ancymidol concentration (16 μM), *fpa1-4* germination remained high (over 60%), while WT germination was reduced to 10%. Taken together, these results indicate that germination, vegetative growth and floral induction of *fpa1-3* and *fpa1-4* are partially insensitive to active GA levels.

Molecular and genetic analyses of fpa1-3 and fpa1-4

Allelism of *fpa1-3* and *fpa1-4* was examined by cross-pollinating the mutants homozygous for LUC and the kanamycin resistance marker with the mutants depleted of the transgene by previous backcrossing to non-transgenic Col-0 WT. LUC activity levels and flowering time of kanamycin-resistant F_1 seedlings were similar to parentals, indicating that *fpa1-3* and *fpa1-4* are allelic. SSLP-based mapping indicated that *fpa1-3* and *fpa1-4* mutations were located on chromosome II, south of the markers nga361 and nga168 (respectively 9 and 1 recombination events out of 48 plants examined). Since the *fpa1* mutation, which confers a late flowering phenotype, maps to this region, allelism was examined by crossing *fpa1-3* and *fpa1-4* to *fpa1-1* (Koornneef *et al.*, 1991). All progeny from both crosses exhibited late flowering, confirming allelism. The *FPA* gene has been recently isolated by positional cloning, and genomic sequencing of the *FPA* transcribed region revealed that *fpa1-3* contains an A-T base change resulting in a nonsense mutation at amino acid 120 of the predicted FPA protein (F. Schomburg and R. Amasino, unpublished results). No mutations were identified in the FPA open reading frame of *fpa1-4*, indicating that this allele may be caused by a flanking mutation affecting expression of the *FPA* gene.

To determine whether *fpa1-3* exhibited altered patterns of GA-related gene expression, mRNA levels of different genes were assessed in rosette leaves and inflorescences with or without application of GA_3 (Figure 6b). This confirmed that *GA5* mRNA in the WT accumulated to

higher levels in the inflorescence than in the rosette, and that GA_3 treatment reduced *GA5* mRNA levels in both tissues (Phillips *et al*., 1995; Xu *et al*., 1995). This pattern was also observed in *fpa1-3*, suggesting that although *fpa1-3* quantitatively effects *GA5* expression, the *fpa1-3* mutation does not qualitatively affect GA feedback regulation of the *GA5* gene. Surprisingly, *GA5* mRNA levels in *fpa1-3* were only slightly higher than in WT, although LUC activity from the *GA5-LUC* reporter was more than 20 times higher in *fpa1-3* than in WT (Figure 3b). RT-PCR quantitation showed that LUC mRNA levels correlated with LUC activity (Figure 6a). Therefore, the difference observed between the endogenous *GA5* and LUC-reporter mRNA levels may reflect differences in the stabilities of the two mRNAs. In any event, the increased levels of *GA5* mRNA in *fpa1-3* were paralleled by increased levels of GA 2-oxidase (Figure 6b). These data suggest that increased GA biosynthesis via *GA5* in *fpa1*-3 results in increased GA metabolism via GA 2-oxidase, which converts bioactive GAs to inactive metabolites (Thomas *et al*., 1999).

Steady state mRNA levels of the GA responsive gene *GASA1 and* the GA regulatory gene *GAI* were also compared in *fpa1-3* and wild type. *GAI* mRNA levels were lower in *fpa1-3* than wild-type rosettes (Figure 6b). Since *GAI* negatively regulates GA responses in vegetative tissues, the decreased *GAI* mRNA level in *fpa1-3* is consistent with the increased *GA5* mRNA levels noted above. Lower *GAI* expression would also be consistent with heightened levels in *fpa1-3* of GA responsive *GASA1* mRNA following GA application (Figure 6b). Taken together, these results indicate that, while the levels of *GAI* and *GASA1* expression are quantitatively different in *fpa1-3*, this mutation does not qualitatively affect GA responses.

Discussion

GA feedback regulation of the GA5 promoter

Levels of bioactive GAs are controlled by feedback regulation of GA biosynthetic and metabolic enzymes. Thus, bioactive GAs enhance the steady-state levels of mRNA of the GA metabolizing GA 2-oxidase (Thomas *et al*., 1999), and reduce the RNA levels of the biosynthetic *GA4* 3β-hydroxylase and *GA5* GA 20-oxidase (Chiang *et al*., 1995; Phillips *et al*., 1995; Xu *et al*., 1995; Yamaguchi *et al*., 1998b). We show here that GA feedback control of *GA5* is mediated, at least in part, via transcription from the *GA5* promoter, and that the required *cis* elements are located within 500 bp of the promoter. However, other or additional levels of control could be mediated by short-lived factors affecting the stability of *GA5* mRNA, as suggested by the difference in *GA5* and *LUC* transcript abundancies observed in wild-type and the *fpa1-3* and *fpa1-3* mutants.

GA5-LUC bioimaging screen

Since the activity of the *GA5-LUC* reporter mimicked that of endogenous *GA5* mRNA, we initiated a screen for mutants altered in LUC expression. To this end, M_2 seedlings were screened after 5 days of germination on plates containing 50 μM GA_3 by imaging the bioluminescence from the *GA5-LUC* reporter. GA_3 was included in the media for several reasons. First, mutants in which repression or GA negative feedback is disrupted would be discernible from WT because it is easier to image plants with higher than normal LUC activity. Second, GA application should partly synchronize germination in a seed population and therefore reduce differences in LUC expression levels due to developmental variations (Thornton *et al*., 1999). Third, although mutants blocked in GA biosynthesis would germinate normally when supplied with GA_3, *GA5-LUC* reporter expression would be repressed in them and they therefore would be easily discarded (Koornneef and van der Veen, 1980; Steber *et al*., 1998). This screen permitted the isolation of 12 recessive mutants, three with lower (*loe1-3*) *and* 9 with higher (*lue1-lue7*, *fpa1-3* and *fpa1-4*) LUC levels than wild type. Three of the high LUC expressing lines were chosen for further characterization because they might be caused by loss of repressive function(s), and because they exhibited potentially GA related growth phenotypes.

The lue1 semi-dwarf mutant

SSLP-based mapping showed that the mutation responsible for the semi-dwarf phenotype of *lue1* is located on the bottom of chromosome I, in a region containing the *GA2 ent*-kaurene synthase whose loss of function results in dwarfing (Yamaguchi *et al*., 1998a). However, allelism tests show that *lue1* is not allelic to *ga2-1*, which is consistent with our finding that repeated GA treatment restores wild-type stature to *ga2-1,* but not to *lue1*. It is also unlikely that *lue1* corresponds to *sly1* (Steber *et al*., 1998) because *sly1* is characterized by failure to germinate in the absence of the *abi1-1* lesion, while *lue1* germination is comparable to wild type. *Lue1* is also probably unrelated to tobacco *RSG* (Fukazawa *et al*., 2000), because *RSG* homologs are not as yet found within the region of chromosome I to which *lue1* maps. In addition, expression of a dominant-negative form of RSG results in a GA-deficient phenotype which can be normalized by GA applications, clearly not the case for *lue1*. *lue1* may thus represent a novel dwarf mutant altered in its sensitivity to GAs.

The fpa1-3 and fpa1-4 late flowering mutants

Two late flowering mutants from separately mutagenized pools were identified by markedly increased *GA5-LUC*

activity, and shown by mapping, allelism tests and genomic sequencing to be caused by alleles of *fpa1*, here designated *fpa1-3* and *fpa1-4*. Since the mutation in *fpa1-3* produces a nonsense mutation early in the predicted FPA protein, the recessive *fpa1-3* is a loss of function allele (F. Schomburg and R. Amasino, unpublished results). A full characterization of FPA will be described elsewhere. Initial quantitation of endogenous GA levels in *fpa1-3* grown under LD show that, compared to WT, *fpa1-3* contains equivalent levels of GA_9 and GA_{53}, but three- to sevenfold higher GA_{19} and 10- to 16-fold higher GA_4 levels (T. Moritz, personal communication). These alterations in GA levels are consistent with overexpression of *GA5* 20-oxidase. For example, the *ga5* deletion mutant has equivalent GA_{19}, lower GA_9 and GA_4, and higher GA_{53} levels than WT (Talon *et al.*, 1990), while plants engineered to overexpress GA 20-oxidase contain increased GA_4 levels (Coles *et al.*, 1999).

FPA is thought to constitutively promote flowering together or in parallel with *FCA*, *LD*, *FVE* and *FY* because their recessive mutations all result in delayed flowering under both long and short days (Fowler *et al.*, 1999; Koornneef *et al.*, 1998). Epistasis analyses indicate that these autonomous pathway genes act in a photoperiod independent manner to negatively regulate expression of *FLC*, which in turn acts to repress flowering (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Genetic and physiological studies indicate that GAs modulate the effects of the autonomous flowering pathway. For example, GA insensitive *gai* and GA deficient *ga1* mutants are late flowering under SD, while *spy*, in which GA signal transduction is constitutively active, flowers early (Jacobsen and Olszewski, 1993). However, it has been difficult to determine whether GA acts via or in parallel with the autonomous pathway because certain late flowering mutants such as *fca* and *fve* flower later than *ga1* in long days (Koornneef *et al.*, 1991). It is clear that expression of the floral meristem identity gene *LFY* is activated by GAs via promoter *cis* elements separate from those mediating *LFY* expression in response to the photoperiod (Blazquez and Weigel, 2000). This indicates that the *LFY* promoter integrates hormonal- and photoperiod-dependent signals controlling flowering. Nonetheless, it is still unclear how the autonomous pathway genes may affect the expression of *LFY*, the floral inducer *FT*, or *TFL*, an *FT* homolog, which acts to repress flowering (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999).

We show here that loss of function of the autonomous pathway gene *FPA* affects GA-dependent processes at the molecular and physiological levels. Models of the role of GA in flowering propose that the vegetative to reproductive transition is mediated both by increases in GA levels, and by differential changes in GA responsiveness of various target tissues (Xu *et al.*, 1997). Recently, two groups have provided evidence that FLC is a negative regulator of GA activity in the apex (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). In the *FLC* over-expressor *flf* mutant, application of active GAs failed to induce flower differentiation, in contrast to wild-type (Sheldon *et al.*, 1999). Moreover, members of the late-flowering mutant group, such as *fpa1*, exhibit increased *FLC* transcript levels, indicating that *FLC* is negatively regulated by the products of these genes. These observations are consistent with our finding that *fpa1-3* and *fpa1-4* show reduced sensitivity to GA levels for floral promotion and during germination, suggesting that FPA modulates a GA signaling pathway through FLC. In *fpa1-3* and *fpal-4*, reduced FPA protein levels result in high FLC mRNA/protein levels, probably leading to repression of the GA signaling pathway(s). In the *fpa* mutants, altered LFY induction by GAs, as well as altered GA feedback regulation of *GA5* and *GA4*, might both contribute to the late-flowering phenotype and the higher *GA5* and *LUC* reporter transcript levels.

Interestingly, SPY, a repressor of GA signaling, interacts at the protein level with GIGANTEA (N.E. Olszewski, personal communication), which regulates photoperiod-dependent flowering (Fowler *et al.*, 1999; Huq *et al.*, 2000). This interaction may be a point of crosstalk between the GA and phytochrome signaling pathways, perhaps *via* SPYs O-acetylglucosamine transferase activity (Thornton *et al.*, 1999). Further work is required to understand how the apparent modulation of GA responses by FPA may be integrated with such other inputs in the control of flowering time.

Experimental procedures

Plant material

Seeds of Arabidopsis ecotypes Col-0 and *Ler* and transgenic plants derived from them were surface-sterilized and germinated on MS medium supplemented with 0.7% agar and 1% sucrose. Plants were grown in a greenhouse for seed amplification. GA-deficient *ga1-1*, *ga2-1* and *ga5* mutants were from the Nottingham Arabidopsis Stock Center. Application of GA (50 μM GA_3, Sigma) was performed weekly on *ga5* and *ga1-1* mutants to obtain fertile plants for crosses. Plant material for bioimaging and reporter assays were grown in Percival (Perry, IA, USA) AR-75 growth cabinets under short day (SD) 10/14 h light/dark or long day (LD) 20/4 h light/dark at 21°/18° for the light/dark cycle and 70% relative humidity. For hormonal treatments, 7-day-old seedlings, grown under constant light, were transferred to fresh MS plates containing 50 μM GA_3 or 4 μM concentrations of the GA biosynthetic inhibitor ancymidol (Sigma). Unless otherwise specified, treatments were performed for 5 days. For translational inhibition assays, 5-day-old-seedlings grown on MS plates were transferred to 50 ml liquid 1/2 × MS in flasks for 1 day prior to treatment. The translational inhibitors cycloheximide (50 μM) and chloramphenicol (50 μM) were added to the medium 2 h before treatment with 50 μM GA_3 for 16 h.

Transgenics expressing fusions of the GA5 promoter to LUC or GUS

5′ upstream sequences of *GA5 (*GA 20-oxidase; cDNA GenBank accession numbers AT2301 and U20901) were PCR amplified using a template from a library of randomly sheared Col-0 genomic DNA (Mundy *et al.*, 1995) and *GA5* cDNA primers (CM1: CAGTTACGGTACAGGTGGTCGG or CM2: GGAGATACCTAGCCA-AAGCTG) and vector primers (JMR2: GGTGACACTATAGAAG-AGCTCG, JMR14: CATGGTTCTCTCCAGAGGTTC, or JMR33: CAGCGCCGCACATGGTACAGCAAG). Two products of 900 and 400 bp from primer combination CM1/JMR2 were digested with *Bam*HI/*Hin*dIII, cloned in pBS SK + (Stratagene), and sequencing revealed them to contain overlapping *GA5* upstream fragments. Linkered fragments were then amplified by 900 bp (primers CM5: GGAAGATCTGAGAGATTATAGTAAAGT/JMR12: AGCGGATAA-CAATTTCACACAGGA) and 500 bp (primers CM5/CM6: CGCGGA-TCCGCGGAATAGTGGTAATTGTCC) of the putative *GA5* promoter. These products were digested with *Bam*HI/*Bgl*II and cloned in the *Bam*HI site of the binary vector vip11 (Millar *et al.*, 1995), upstream of the omega leader sequence (Gallie *et al.*, 1987), the firefly Luciferase (*LUC*) gene and the rubisco small subunit E9 terminator sequence, to generate the *GA5-LUC* and *GA5*-900-*LUC* transgene constructs. Alternatively, the omega leader/*LUC* cassette was excised with *Hin*dIII/*Sst*I and replaced by the *E. coli uidA gene* β-glucuronidase (*GUS*; Jefferson *et al.*, 1987) to generate constructs *GA5*–500-*GUS* and *GA5*-900-*GUS* for histochemical analyses of *GA5* expression. Transgene constructs were mobilized into *Agrobacterium tumefaciens* strain ABI by electroporation prior to plant transformation by vacuum infiltration (Bechtold and Pelletier, 1998). Infiltrated plants were allowed to self, T_1 seedlings selected on MS plates with 50 mg l^{-1} kanamycin, and transferred to soil. Approximately 40 lines for each construct were analysed further.

Reporter assays

Histochemical localization of GUS activity was performed according to Jefferson *et al.* (1987). Equipment and protocols for LUC bio-luminescence imaging have been described previously (Michelet and Chua, 1996; Raventos *et al.*, 2000). In brief, images were taken of 2000 5-day-old WT seedlings germinated on plates with MS or MS suplemented with 50 μM GA_3. The average grey value (AGV) of all seedlings as objects were measured and data transferred to a spreadsheet. None of the GA-treated WT seedlings had AGVs above 32 units, and therefore all objects between 10 and 32 AGV were given a blue filter color. A safety margin of 10 units was incorporated such that only objects with an AGV greater than 42 units were given a red filter color and would be identified as LUC overexpressing plantlets. Superimposition of the red filter image onto the bright-field image enabled identification of LUC super expressing (*lue*) mutants. Similarly, isolation of LUC low expressing (*loe*) mutants was performed by applying a filter (15–100 AVG) enabling detection of seedlings whose AGV was below 15 units.

Quantitative *in vitro* LUC assays were performed on plant extracts using a Victor2 1420 counter with injector (Wallac, Allerød, Denmark). Plant samples were homogenized in 100 mM KH_2PO_4/K_2HPO_4 pH 7.8 and 1 m DTT, centrifuged, and supernatants supplemented with 25 mM tricine pH 7.8, 15 mM $MgCl_2$, 5 mM ATP, 0.5 mg ml^{-1} BSA. A 200 μl sample was then transferred to 96-well white microtiter plates (Costar, NY, USA). The reaction was carried out by automatic injection of 100 μl 0.5 mM luciferin (JBL/Promega, Madison, WI, USA), followed by 1 sec shaking and a 5 sec luminescence measurement. Each data point represents the mean value of six independent measurements from duplicate experiments.

Mutagenesis and mutant isolation

The T_4 *Col-0* line carrying *GA5-LUC*, here called WT transgenic, was selected for mutagenesis as progeny analysis showed it homozygous for a single locus carrying the kanamycin resistance marker with the *GA5-LUC* transgene. This line also expressed sufficient LUC activity for bioluminescence imaging and showed appropriate levels of repression of LUC activity following GA treatment. 25 000 bulked seeds were γ-irradiatiated (60 kRad) from a cobalt^{60} source (RISØ Industrial Irradiation, Denmark). M_1 seeds were bulked in 20 pools of 1250 seeds each and propagated in a long day greenhouse. Pools were harvested individually.

100 000 M_2 seedlings were screened for LUC expression by bioluminescence imaging. Seeds were plated on Ø15 cm plates (1000 seeds per plate) containing 50 mg l^{-1} kanamycin and 50 μM GA_3, stratified 48 h at 4°, then transferred to a 21° chamber under constant light. *In vivo* LUC activity was measured on 5-day-old seedlings. Seedlings exhibiting reduced or increased levels of LUC activity were transferred to fresh plates and re-screened 2 days later for altered LUC expression levels compared to the WT transgenic. Eighty plantlets initially identified as *lue* and *loe* putative mutants were transferred to soil and grown under LD conditions. Plants were allowed to self and LUC expression re-examined in progeny. Three *loe* and nine *lue* putative mutants were thus confirmed as impaired in LUC reporter regulation.

RNA analysis

Total RNA was extracted using the RNAgent kit (Promega) and Poly A^+ RNA isolated using the PolyA-tract kit IV (Promega). Poly A RNA samples were size-fractionated on standard formaldehyde gels and blotted onto Hybond-N + membranes (Amersham). mRNA levels of genes involved in GA biosynthesis (*GA5, GA4*), GA metabolism *(GA 2-oxidase),* GA-signaling *(RGA, GAI)* or GA-regulation (*GASA-1*) were investigated by Northern hybridization. Ribonucleic ^{32}P-CTP antisense probes were synthesized using T7 RNA polymerase (Ribokit, Promega) from partial cDNA 3′ sequences cloned in the pGEM-Teasy vector (Promega). Primer sequences are available upon request. Hybridization and washing conditions were performed as recommended by the manufacturer. As a control, radioactive signals on all membranes were quantified by phospho-imagery (Molecular Dynamics, Sunnyvale, CA, USA) by comparison to signals from subsequent hybridization with an *EF1-α* probe *(*Axelos *et al.*, 1989*).*

For RT-PCR, total RNA was treated with RNase-free DNase (RQaseI, Promega) at 0.5 unit per μg RNA. Before reverse transcription, PCRs were performed to assess contamination with genomic DNA. Oligo(dT) primer (2 μg) was annealed to total heat denaturated RNA (50 μg) and first-strand cDNA synthetized at 37° for 1 h using 1 U M-MuLV reverse transcriptase (Stratagene) per μg RNA, in the presence of 0.6 U RNase inhibitor (RNasin, Promega) per μg RNA. First strand cDNAs were used as templates for the PCR amplification of *LUC* (400 bp) and *EF1-α* (474 bp). *EF1-α* was used as an internal control for PCR amplification. PCR conditions were as follows: 30 cycles of 92°, 30 sec; 62°, 30 sec; 72°, 40 sec. Because of differences in cDNA abundancies between *LUC* and *EF1-α*, the first 10 cycles were performed in the absence of *EF1-α* primers.

Genetic analysis

Lue1, *fpa1-3* and *fpa1-4* were crossed with ecotype *Ler*, F_1 plants allowed to self, and F_2 seeds collected. Homozygous mutants that segregated in the F_2 population were selected by LUC imaging or visible growth phenotypes. Genomic DNA was extracted from each plant and mapping carried out as previously described (Bell and Ecker, 1994) with recombination data analysed at http://www.msu.edu/~venkata1/sslPfull.htm. The *GA5-LUC* reporter transgene was mapped to chromosome 5, in the vicinity of AthS0262 (no recombination event out of 30 plants).

Acknowledgements

We thank Thomas Moritz for performing preliminary measurements of the endogenous GA levels from the *fpa1-3* and *fpa1-4* mutants, and Suksawad Vongvisuttikun for excellent technical assistance. This work was supported by European Union Grants CT96-0062 and CT96-0621 to J.M. and by a postdoctoral Marie Curie Research Training Grant to T.B.

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