

# FPF1 Promotes Flowering in Arabidopsis

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**We have characterized the gene *flowering promoting factor1* (*FPF1*), which is expressed in apical meristems immediately after the photoperiodic induction of flowering in the long-day plants mustard and Arabidopsis. In early transition stages, expression is only detectable in the peripheral zone of apical meristems; however, later on, it can also be found in floral meristems and in axillary meristems that form secondary inflorescences. The *FPF1* gene encodes a 12.6-kD protein that has no homology to any previously identified protein of known function. Constitutive expression of the gene in Arabidopsis under control of the cauliflower mosaic virus 35S promoter resulted in a dominant heritable trait of early flowering under both short- and long-day conditions. Treatments with gibberellin (GA) and paclobutrazol, a GA biosynthesis inhibitor, as well as crosses with GA-deficient mutants indicate that *FPF1* is involved in a GA-dependent signaling pathway and modulates a GA response in apical meristems during the transition to flowering.**

## INTRODUCTION

After seed germination, morphogenesis in higher plants originates from shoot and root apical meristems. After a certain phase of vegetative growth, the transition to flowering is brought about by the concerted action of endogenous and environmental factors that synchronize plants of a given species to ensure their reproductive development under optimal conditions. The most important environmental factors that control floral induction are low temperatures (vernalization) and the length of the daily light period. Whereas the vernalization temperature is recognized by the shoot apical meristem, flower induction by a favorable day length takes place in the leaves. In photoperiodic plants that are growing under the appropriate day length, a flowering stimulus is released from the leaves and transported to the apical meristem (Bernier et al., 1993). If the apical meristem is competent to respond to this signal, the vegetative meristem becomes either an inflorescence or a floral meristem, from which floral development continues (McDaniel et al., 1992).

One of the first known responses in apical meristems after the induction of flowering is the activation of the floral meristem identity genes *LEAFY* (*LFY*) (Weigel et al., 1992) and *APETALA1* (*AP1*) (Mandel et al., 1992) in Arabidopsis and their homologs in Antirrhinum, *FLORICAULA* (*FLO*) (Coen et al., 1990) and *SQUAMOSA* (*SQUA*) (Huijser et al., 1992). MADS box genes of unknown function that are activated before the floral meristem identity genes in the apical meristems of Arabidopsis and mustard in response to floral induction have recently been identified (Mandel and Yanofsky, 1995a; Menzel

et al., 1996). The analysis of mutants of Arabidopsis that flower earlier or later than wild-type plants has resulted in complex models of interacting genetic functions (Martinez-Zapater et al., 1994). The first two genes cloned from late-flowering mutants have been shown to encode regulatory proteins that are constitutively expressed. *LUMINIDEPENDENS* (*LD*) appears to affect light perception (Lee et al., 1994), whereas *CONSTANS* (*CO*) is required to promote flowering under long-day (LD) conditions (Putterill et al., 1995). It has been shown that constitutive expression of *LFY* (Weigel and Nilsson, 1995), *AP1* (Mandel and Yanofsky, 1995b), and *CO* (Simon et al., 1996) leads to earlier flowering of Arabidopsis plants, both under short-day (SD) and LD conditions. None of these early flowering transgenic lines flower immediately after germination, and all frequently develop terminal flowers. These results indicate that additional genetic functions are required for the induction of flowering.

It has been shown repeatedly that treatments with gibberellins (GAs) can cause flower formation in different species, especially in LD plants that form rosettes under noninductive conditions (Zeevaart, 1983). Furthermore, it is known that in some LD plants, the amount of endogenous GAs is increased by transferring these plants into inductive environments (reviewed in Metzger, 1995). GA-deficient and GA-insensitive mutants of Arabidopsis have provided additional evidence that GAs are required for flowering (Wilson et al., 1992); however, whether a GA signaling pathway is activated in the apical meristem in the transition to flowering has not been shown. Previously, we have applied a subtractive hybridization technique to identify genes that respond to photoperiodic induction in the apical meristem of the LD plant mustard (Melzer et al., 1990). Several apex-specific genes could be identified, but only one gene (*pSFD5.04*,

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now designated *FPF1* for [flowering promoting factor1) was activated specifically after the induction of flowering. In this study, we have molecularly characterized the *FPF1* genes in mustard and Arabidopsis and show that the gene product mediates a GA response in the apical meristem, which leads to earlier flowering in transgenic plants constitutively expressing *FPF1*.

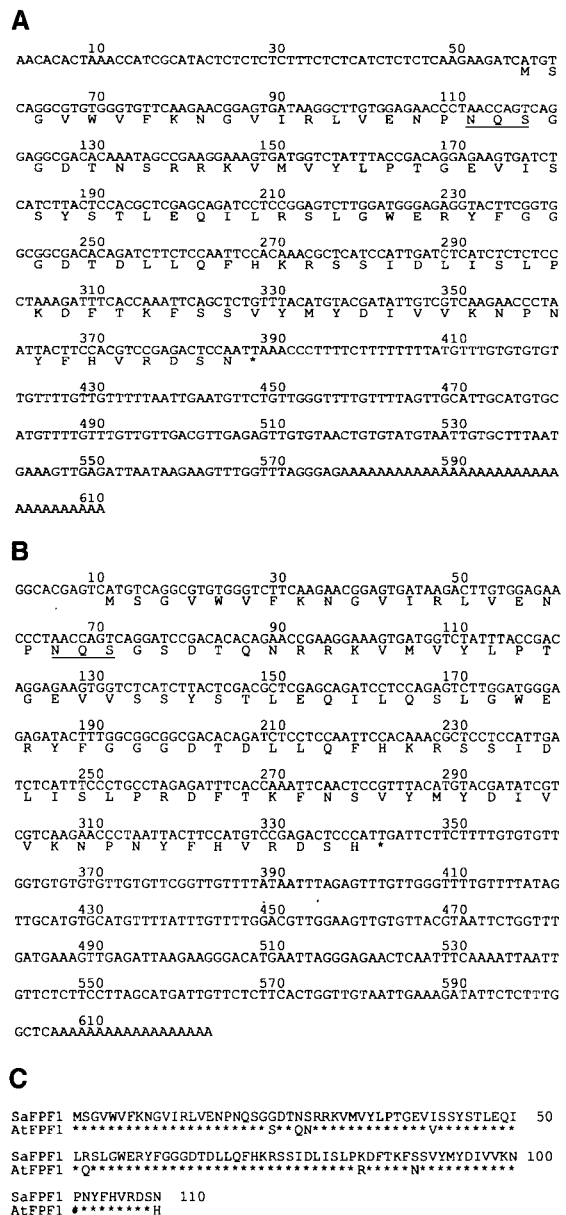
**RESULTS**

**cDNA Cloning**

A cDNA fragment of *FPF1* (formerly *pFSD5.04*) was isolated initially by subtractive hybridization and differential screening (Melzer et al., 1990). To isolate a full-length cDNA, we screened a cDNA library synthesized from mustard apices on the seventh day after floral induction. For this purpose, the day-length dependence of floral induction was exploited to synchronize flower formation in a large population of mustard plants. Meristems from ~500 plants were dissected, poly(A)<sup>+</sup> RNA was isolated, and a cDNA library was generated as described by Menzel et al. (1996). From 500,000 phages of an unamplified library, seven phages with cDNA insertions of different lengths were selected after hybridization with the *FPF1* fragment.

The longest *FPF1* cDNA isolated is 610 bp long with a 330-nucleotide open reading frame that encodes a 110-amino acid protein with a calculated molecular mass of 12.6 kD (Figure 1A). The protein is basic with a pI of 9.8, is slightly serine rich, and with the amino acids NQS contains a possible N-glycosylation site in the N-terminal part. To study the *FPF1* gene in Arabidopsis, a homologous *FPF1* cDNA was identified in a cDNA library of young inflorescences. As shown in Figure 1B, the 622-bp-long *AtFPF1* cDNA also contains an open reading frame of 330 nucleotides, and the encoded protein shows an identity of 92.7% with the mustard protein (Figure 1C). No homologous proteins with known functions could be identified in the data banks except for three expressed sequence tags from Arabidopsis. One of the expressed sequence tags (40B10T7) is identical to *AtFPF1*, whereas the other two (95G12T7 and 98K16T7) are identical and showed 81% identity at the nucleotide level to *AtFPF1*. This second gene is not expressed in the apical meristem upon floral induction (T. Kania and S. Melzer, unpublished data).

To investigate whether mutants that might be defective in the *FPF1* gene had already been described in the literature, we mapped the gene in Arabidopsis. With the recombinant inbred lines of Lister and Dean (1993), we mapped *FPF1* to chromosome 5, at 1 centimorgan above the molecular marker g4556. No mutation that affects flowering time or plant growth as described later was found at or close to this location in the mapping data of the Arabidopsis database at Stanford University (Stanford, CA).



**Figure 1.** Sequence Analysis of *FPF1* cDNAs from Mustard and Arabidopsis.

- (A) Nucleotide and deduced protein sequence of the mustard *FPF1* cDNA.
- (B) Nucleotide and deduced protein sequence of the Arabidopsis *FPF1* cDNA.
- (C) Comparison of the deduced amino acid sequences of the *FPF1* gene product from mustard (SaFPF1) and Arabidopsis (AtFPF1). Asterisks indicate identical amino acids, and a putative N-glycosylation site is underlined. EMBL accession numbers are Y11987 for SaFPF1 and Y11988 for AtFPF1.

### RNA Gel Blot Analysis

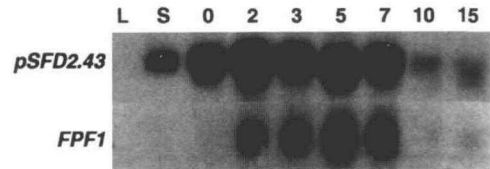
To determine where the *FPF1* gene is expressed in mustard plants, RNA gel blot analysis was used. We analyzed RNA extracted from different plant organs and from the apical buds of 2-month-old vegetative plants as well as of plants that were transferred to LD conditions. As shown in Figure 2, transcripts of *FPF1* could not be detected in fully developed leaves, in the upper first centimeters of the shoot, or in apical buds of vegetative plants. The steady state mRNA level increased in apical buds upon induction of flowering until day 7 after the start of the LD treatment. The sharp decrease at day 10 was not due to a downregulation of the gene but rather to a dilution of the *FPF1* mRNA with high amounts of mRNA of older flower buds. *FPF1* transcripts were not detectable by RNA gel blot analysis in excised 3-mm root tips of 4-day-old mustard seedlings or in whole roots 10 days after germination (data not shown).

### In Situ Hybridization

To determine where the *FPF1* gene is expressed spatially in mustard and Arabidopsis, we performed in situ hybridizations with apical buds of vegetative plants that were grown under SD conditions and with plants that were induced to flower by LD conditions. As shown in Figure 3A, in mustard, *FPF1* mRNA was not detectable in vegetative meristems. After the start of the LD treatment, *FPF1* mRNA appeared in the peripheral zone of the apical meristem (Figure 3B). This expression was maintained during inflorescence development, and the gene was also expressed in emerging floral meristems but not in older floral buds in which the floral meristem had been consumed (Figure 3C). In Arabidopsis, the expression pattern was similar to that observed in mustard (Figures 3D to 3F). Transcripts of the *FPF1* gene were not detectable by in situ hybridization in apical meristems of vegetative plants (Figure 3D). Four days after the beginning of the LD treatment, *FPF1* mRNA was present in the peripheral zone of the meristem where floral primordia emerge, but this was not as prominent as in mustard (Figure 3E). At later stages, expression was maintained in the peripheral zone and was detectable in emerging floral primordia and in the anlagen of stamens (Figure 3F).

### Constitutive Expression of *FPF1* in Arabidopsis

The possible function of the *FPF1* gene was analyzed by constitutively expressing the mustard and Arabidopsis cDNAs in transgenic Arabidopsis plants. To do this, the coding region of *FPF1* was amplified via polymerase chain reaction and linked to the cauliflower mosaic virus 35S promoter (Odell et al., 1985). The Arabidopsis ecotype Columbia (Col) was transformed with either the mustard (*SaFPF1*) or the Arabidopsis *FPF1* (*AtFPF1*) cDNA and the Landsberg *erecta* (*Ler*) ecotype



**Figure 2.** RNA Gel Blot Analysis of *FPF1* Gene Expression in Mustard.

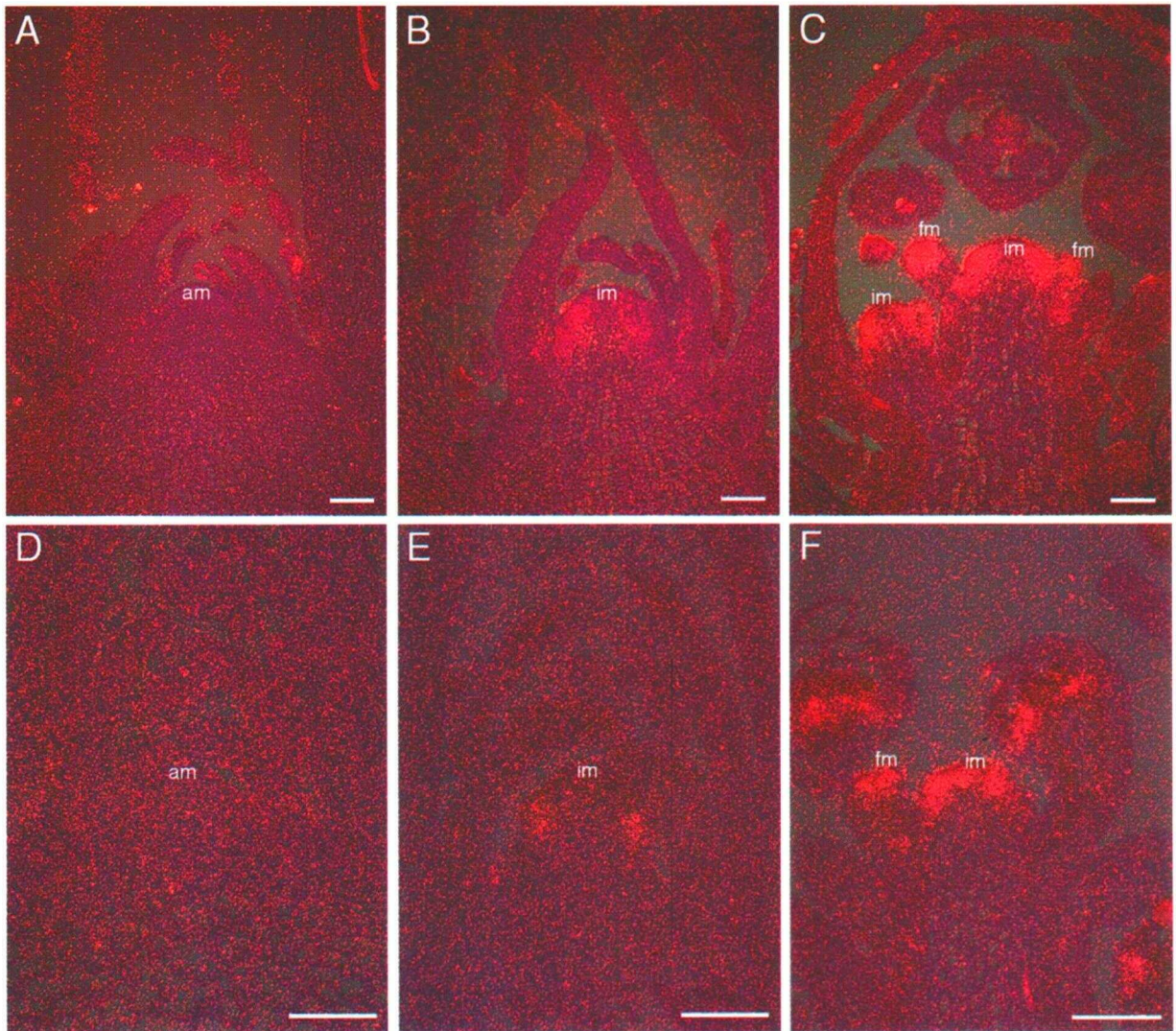
Poly(A)<sup>+</sup> RNA (2 μg per lane) from mature leaves (L), excised 1-cm apical shoots (S), and 0.3-mm apices of vegetative plants (0) and of plants grown under different inductive LD periods (2, 3, 5, 7, 10, and 15 days) was transferred to nitrocellulose membranes after electrophoresis in denaturing formaldehyde gels. As a control, *pSFD2.43*, which is expressed in apical buds before and after floral induction (Melzer et al., 1990), was used.

with only the *AtFPF1* cDNA by vacuum infiltration of inflorescences (Bechtold et al., 1993). We obtained 20 Col lines constitutively expressing the *SaFPF1* cDNA in addition to six Col lines and eight *Ler* lines expressing the *AtFPF1* cDNA. One Col line expressing the *SaFPF1* cDNA, all lines overexpressing *AtFPF1* in Col, and one *Ler* line were analyzed in greater detail. Plants were grown under LD (16-hr light and 8-hr darkness) or SD (8-hr light and 16-hr darkness) conditions. All transgenic plants expressing either the *SaFPF1* or the *AtFPF1* cDNA were altered in the duration of the vegetative phase.

As shown in Figures 4A and 4B, the transgenic Col line *AtFPF1-4C* (which contains a single transgene insertion) flowered earlier and produced fewer leaves than did the control plants under both LD and SD conditions. The organization of the flowers, the fertility, and the seed set were the same as in wild-type control plants. However, the architecture of the transgenic plant had changed. Whereas, on the inflorescence stem of wild-type plants, cauline leaves without petioles were inserted, the transgenic plant had rosettelike leaves with petioles at the same positions (Figure 4C). This was because of an internode elongation between rosette leaves, which caused bolting of the plants before rosette leaf production was terminated. Compared with the wild-type plants, the transgenic plants had fewer rosette leaves and the number of cauline leaves was increased. However, the overall leaf number was reduced under both day-length conditions.

For a comparison of the wild-type and the transgenic plants, we scored the leaf number of 12 individual plants from each transgenic line and wild-type plants under both photoperiods. As shown in Figure 5, the reduction of the rosette leaf number and hence the time to flowering varied between the different transgenic lines and ecotypes. The transgenic Col line expressing the mustard *FPF1* cDNA (*SaFPF1-3C*), which was selected as the earliest flowering line of 20 independent transformants, showed a phenotype indistinguishable from that of the transgenic Col plants expressing the Arabidopsis transgene. Under LD conditions,





**Figure 3.** Detection of *FPF1* RNA by in situ hybridization in apical buds of mustard and Arabidopsis.

In (A) to (C), mustard was used; in (D) to (F), Arabidopsis plants were used.

(A) No expression is visible in apical buds of vegetative plants.

(B) After 2 days under LD conditions, expression of *FPF1* is visible in the peripheral zone of the apical meristem.

(C) At day 7, expression is maintained in the peripheral zone of the apical meristem and is visible in floral meristems and in a secondary inflorescence meristem in the axil of the last formed leaf.

(D) No expression above the background can be seen in apical meristems of Arabidopsis plants grown under SD conditions.

(E) In apical meristems of plants that were transferred for 4 days to LD conditions, *FPF1* transcripts are visible in the peripheral zone where floral primordia emerge.

(F) After 10 days in LD conditions, floral buds are visible on bolting plants. *FPF1* expression is confined to the peripheral regions of the apical meristem and to floral meristems. After sepal development, the expression in floral buds is restricted to the anlagen of stamens.

am, apical meristem; fm, floral meristem; im, inflorescence meristem. Bars = 100  $\mu$ m.

the rosette leaf number in the earliest flowering Col lines (*SaFPF1-3C*, *AtFPF1-1C*, *AtFPF1-4C*, and *AtFPF1-6C*) was reduced from 12 to approximately seven, and the plants had two more cauline leaves; however, the total leaf number was reduced from 15 to 10 leaves (Figure 5A). In SD conditions,

we observed up to 50% reduction in rosette leaf number, and the cauline leaf number increased from 10 to 17 in the earliest flowering Col lines (Figure 5B). The various degrees in the reduction of leaf number correlate with the expression of the transgene. Whereas the RNA level of the transgene





**Figure 4.** Comparison of Wild-Type and Constitutively *PPF1*-Expressing Arabidopsis Plants.

**(A)** Four-week-old plants grown in LD conditions. The wild-type control is still in the vegetative stage (left), whereas the *AtPPF1-4C* plant has opened its first flowers (right).

**(B)** Eight-week-old plants grown under SD conditions. In the wild-type plant (left), flowering is not initiated, whereas the *AtPPF1-4C* plant (right) has bolted and is at anthesis. Leaves at the inflorescence stem of the transgenic plant have petioles, and all leaves are elongated.

**(C)** The 10-week-old wild-type plant (left) bears cauline leaves without petioles on the inflorescence stem. In contrast, the 8-week-old

was highest in lines *AtPPF1-1C*, *AtPPF1-4C*, and *AtPPF1-6C*, it was lowest in line *AtPPF1-2C* and intermediate in lines *AtPPF1-3C* and *AtPPF1-5C* (data not shown).

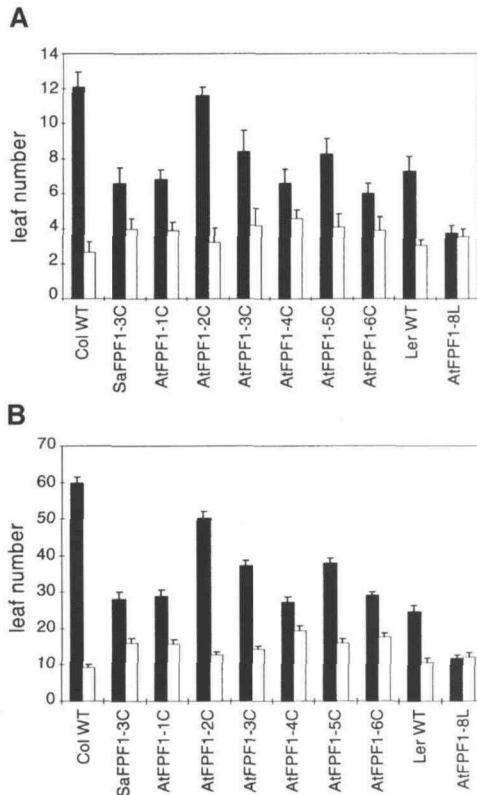
Because most of the flowering time mutants and mutants that are affected in GA biosynthesis were generated in the *Ler* background (Koornneef and Van der Veen, 1980; Koornneef et al., 1991), we also transformed *Ler* plants with the *AtPPF1* cDNA under the control of the cauliflower mosaic virus 35S promoter. Of eight independent transgenic lines, we selected the earliest flowering line with a single transgene insertion for further studies. The *Ler* wild-type plants flowered earlier than did those of *Col* ecotype under both SD and LD conditions (Figures 5A and 5B); however, in the transgenic line *AtPPF1-8L*, the time to flowering was further shortened under both light regimes. In LD conditions, the rosette leaf number was reduced from eight to four leaves in the transgenic plant, and the cauline leaf number was increased by two leaves (Figure 5A). In SD conditions, the *Ler* wild-type plants generated 24 rosette leaves before the floral transition. In the *AtPPF1-8L* line, the leaf number was again reduced by 50%. Compared with the transgenic *Col* plants, the cauline leaf number increased only slightly from nine to 12 cauline leaves in the *Ler* transgenic plants (Figure 5B).

Constitutive expression of antisense constructs of *AtPPF1* in 78 transgenic Arabidopsis lines did not lead to a visible phenotype. This could have been due to a genetic redundancy, or plants with an antisense downregulation of *PPF1* may not be viable.

#### Early Flowering as a GA Response

In addition to the shortening of the time to flowering, we saw some other effects of *PPF1* overexpression in the transgenic plants. Seeds of the transgenic plants germinated earlier, and the hypocotyls of the seedlings were twice as long as those of the wild-type plants. As determined by scanning electron microscopy and as shown in Figure 6, this difference in length is due to an elongation of the hypocotyl cells in the transgenic plants (Figures 6A and 6B). Their rosette leaves were also larger and had longer petioles than those of the wild-type plants. Through the elongation of internodes, the overall architecture of the plant was changed (Figure 4C). These pleiotropic effects are similar to those observed in the *spindly* (*spy*) mutant, which shows a constitutive activation of GA signal transduction (Jacobsen and Olszewski, 1993), and the *phyB* mutant (Reed et al., 1996), in which the lack of phytochrome B leads to an enhanced responsiveness to GAs.

*AtPPF1-4C* transgenic plant (right) shows a gradual increase of internode length, which leads to petioled leaves at the inflorescence stem. Both plants were grown under SD conditions.



**Figure 5.** Leaf Number of Wild-Type and Transgenic Arabidopsis Plants.

The leaves of 12 wild-type (WT) and transgenic Arabidopsis plants were counted once a week. Rosette leaves (solid bars) and cauline leaves (open bars) are depicted separately. The leaf numbers of wild-type plants from the Col and Ler ecotypes are compared with the leaf numbers of the corresponding *FPF1*-overexpressing lines. Under both LD and SD conditions, the rosette leaf number of the transgenic plants is reduced compared with that of the wild-type plants, whereas the cauline leaf number is increased.

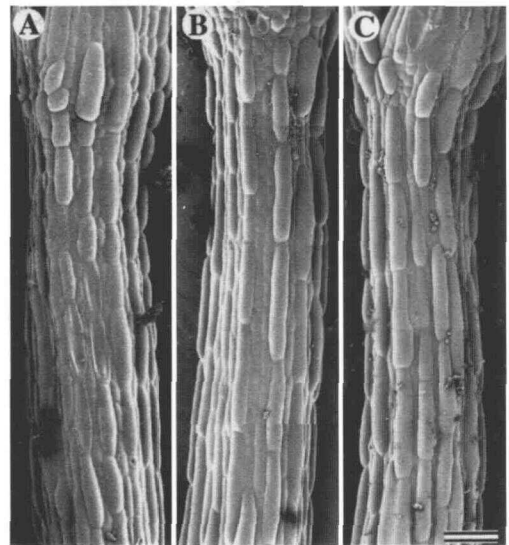
(A) Plants grown under LD conditions.

(B) Plants grown under SD conditions.

Wild-type plants grown on agar plates with Murashige and Skoog salts (Sigma) in the presence of 10  $\mu\text{M}$   $\text{GA}_3$  showed a similar cell elongation in the hypocotyl, as observed in the *FPF1*-overexpressing plants (Figure 6C). To analyze the possible involvement of a GA pathway in the *FPF1*-mediated flowering responses in the transgenic plants, we treated wild-type plants of the Col ecotype and the transgenic line *AtFPF1-4C* under SD conditions after germination with either water or 100  $\mu\text{M}$   $\text{GA}_3$  once a week. The  $\text{GA}_3$ -treated plants resemble *FPF1*-overexpressing plants to a great extent. As shown in Table 1, the  $\text{GA}_3$ -treated wild-type plants had fewer rosette leaves and more cauline leaves and flowered earlier than did the wild-type control plants. Compared with the  $\text{GA}_3$ -treated wild-type Col plants,

the rosette leaf number of the *AtFPF1-4C* plants was more reduced. When *AtFPF1-4C* plants were treated with  $\text{GA}_3$ , the decrease in rosette leaf number and the increase in cauline leaf number were additive (Table 1), and the  $\text{GA}_3$ -treated transgenic plants flowered even earlier than did the untreated transgenic plants or wild-type plants sprayed with  $\text{GA}_3$ .

To further determine whether a GA-dependent pathway is involved in the establishment of the *FPF1* overexpression phenotype, we used the GA biosynthesis inhibitor paclobutrazol, which inhibits monooxygenases that are involved in the oxidation of *ent*-kauren to *ent*-kaurenoic acid (Rademacher, 1991; Jacobsen and Olszewski, 1993), to block the synthesis of GAs under SD and LD conditions. For this experiment, plants were transplanted into individual pots 10 days after sowing and then watered with 37 mg/L paclobutrazol once a week. As shown in Figure 7A, paclobutrazol-treated plants in SD conditions showed a typical dwarf phenotype of plants lacking GAs and produced no visible floral buds after 3 months. Constitutive expression of the *FPF1* gene did not bypass the block in floral development caused by the GA biosynthesis inhibitor. Thus, under SD conditions, GA synthesis is a limit-



**Figure 6.** Comparison of Epidermal Cells from the Hypocotyls of Wild-Type and Transgenic Plants Grown on Murashige and Skoog Medium.

(A) Hypocotyl segment from a 10-day-old wild-type seedling.

(B) Hypocotyl segment from a 10-day-old *AtFPF1-4C* transgenic seedling.

(C) Hypocotyl segment from a 10-day-old wild-type seedling grown in the presence of 10  $\mu\text{M}$   $\text{GA}_3$ .

As determined by scanning electron microscopy, the epidermal cells of the hypocotyl segments of the transgenic (B) and wild-type seedlings grown on medium with  $\text{GA}_3$  (C) are elongated compared with those of the wild-type seedling grown in the absence of  $\text{GA}_3$  (A). Bar in (C) = 100  $\mu\text{m}$  for (A) to (C).

**Table 1.** Leaf Number of Wild-Type and Transgenic Plants Treated with GA<sub>3</sub> or Water under SD Conditions

Plant <sup>a</sup>	Rosette Leaves	Cauline Leaves
Col WT	59.8 ± 1.6	9.3 ± 0.9
Col WT + GA <sub>3</sub>	34.3 ± 1.2	17.6 ± 1.2
<i>AtFPF1-4C</i>	28.6 ± 1.8	16.8 ± 1.1
<i>AtFPF1-4C</i> + GA <sub>3</sub>	14.5 ± 1.7	22.9 ± 1.4

<sup>a</sup>WT, wild type.

ing factor for the *FPF1* response in the transgenic plants. In LD conditions, however, after treatment with paclobutrazol, the transgenic *AtFPF1-4C* plants were able to bolt and to develop an inflorescence, whereas the development of the inflorescence of the wild-type plant was retarded (Figure 7B).

Having the *FPF1* transgene in the *Ler* background, we were able to cross known recessive Arabidopsis mutants that are impaired in GA biosynthesis with the transgenic lines. We used the *ga2-1* mutant, which is blocked in the *ent*-kaurene synthetase B, and the *ga4-1* mutant, which shows reduced levels of 3 $\beta$ -hydroxylation (Finkelstein and Zeveaart, 1994). The *ga2-1* mutant is not totally blocked in GA synthesis but needs exogenous GAs for germination and has a typical dwarf phenotype. The *ga4-1* mutant is leakier and does not need additional GAs for germination (Koornneef and Van der Veen, 1980). Plants from a segregating F<sub>2</sub> population of a cross between *ga2-1* and the *AtFPF1-8L* line grown under SD conditions are shown in Figure 7C. Because the plants were germinated on Murashige and Skoog plates with 10  $\mu$ M GA<sub>3</sub> for 5 days, the segregating wild-type plants flowered earlier than did plants germinated without GA<sub>3</sub>.

The *FPF1* transgenic line with a wild-type background flowered at 5 weeks after germination, whereas the wild-type plants were still in a vegetative stage. Similarly, homozygous *ga2-1* plants and *ga2-1* mutants containing the *FPF1* transgene showed no signs of floral induction at this stage. Both of these plants were dwarfed and dark green in color, but the leaves of the transgenic *ga2-1* plant were more upright. This became more evident 2 weeks later, as shown in Figure 7E. After 10 weeks of growth, both plants showed floral buds in the center of the rosette. A similar cross was made between the *ga4-1* mutant and the *FPF1*-overexpressing line *AtFPF1-8L*. After 5 weeks of growth under SD conditions, the segregating transgenic plant with the wild-type background flowered, whereas the wild-type plant still generated rosette leaves. The homozygous *ga4-1* mutant carrying the *FPF1* transgene flowered too, but it was delayed compared with the wild-type plant with the transgene (Figure 7D). Two weeks later, the *ga4-1* mutant expressing the *FPF1* transgene had developed a stunted inflorescence, whereas the *ga4-1* mutant without the transgene had developed floral buds but no visible inflorescence at this stage (Figure 7F).

To determine whether the GA-dependent phenotype of the transgenic plants could result from an altered responsiveness to GAs, we analyzed further the germination rate of seeds from wild-type and transgenic plants (*AtFPF1-4C*). As shown in Figure 8, no germination occurred on germination medium containing 37 mg/L paclobutrazol. In the presence of different amounts of GA<sub>3</sub>, germination of the transgenic seeds started at a concentration of 10<sup>-4</sup> M GA<sub>3</sub>, whereas the seeds of wild-type plants germinated at a 10-fold higher concentration.

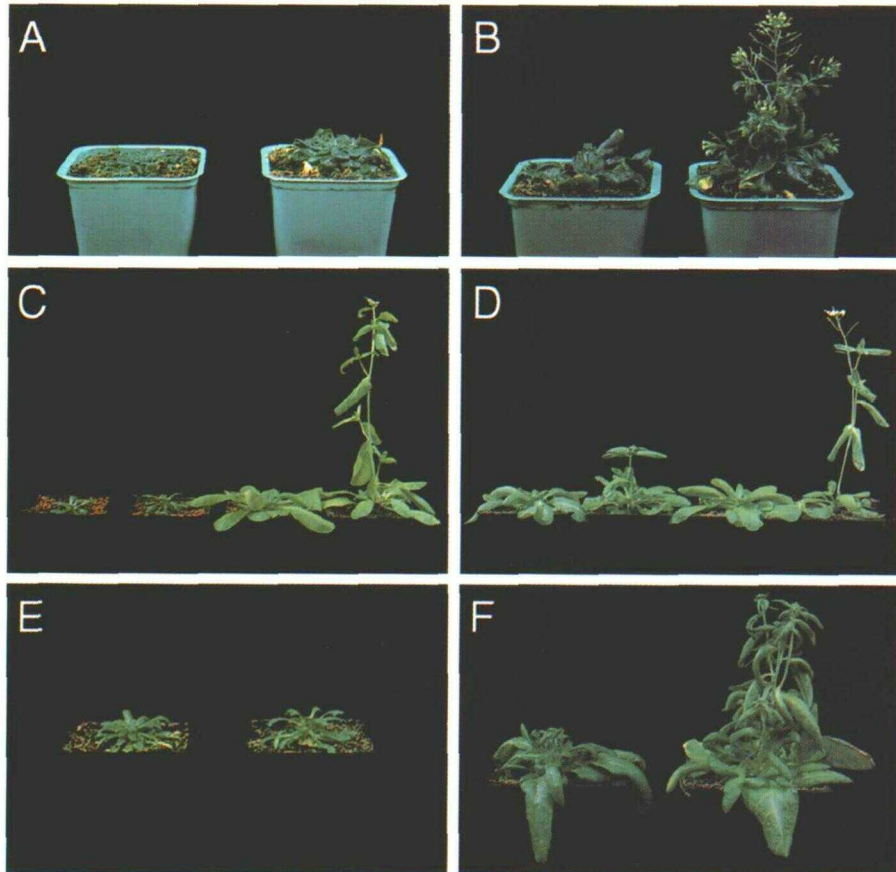
## DISCUSSION

The transition to flowering is controlled by external signals, such as temperature and light (Bernier et al., 1993), and endogenous factors, such as the phytohormone GA (Zeevaart, 1983). These signals interact with flowering time genes in Arabidopsis, of which *CO* and *LD* have been cloned (Lee et al., 1994; Putterill et al., 1995). It has been shown that upon constitutive expression of the flowering time gene *CO*, activation of the meristem identity gene *LFY* and the *TERMINAL FLOWER* gene is rapidly initiated, but activation of *AP1* is not; it seems to need the expression of additional genes for activation (Simon et al., 1996). No mutants completely deficient in flowering have been identified in Arabidopsis, and it has to be assumed that either such mutants are not viable (Weigel, 1995) or that until now not all genes that play a role in the transition to flowering have been identified with known mutants.

In this study, we describe a novel gene, *FPF1*, that is activated soon after floral induction of mustard and Arabidopsis. Together with *LFY* (S. Melzer, unpublished results), *SaMADSA*, and *B* (Menzel et al., 1996), *FPF1* is among the earliest expressed genes that are induced specifically in the apical meristem of mustard after floral induction. In mustard and Arabidopsis, the *FPF1* gene is first expressed in the peripheral zone of the apical meristem, from which the floral primordia differentiate. One of the first changes in the apical meristem of mustard on transition to flowering is an increase in and a synchronization of the mitotic frequency in the peripheral zone (see Bernier, 1988). The expression of *FPF1* in the peripheral zone correlates with these first changes in the apical meristem. The upregulation of *FPF1* expression could merely be a consequence of metabolic changes after flower induction, without necessarily being a causal part of the mechanisms that control flower formation. However, the observed precocious flowering of transgenic plants constitutively expressing the *FPF1* gene suggests that this gene might be involved in early steps of the transition to flowering.

The phenotype of transgenic Arabidopsis plants constitutively expressing the *FPF1* gene resembled that of wild-type plants treated with GAs. This hormone has long been thought to be involved in floral induction. As first shown by Lang (1956) in henbane (*Hyoscyamus niger*) and Langridge (1957) in Arabidopsis, plants treated with GA<sub>3</sub> flower earlier





**Figure 7.** Comparison of GA Responses.

(A) Paclobutrazol-treated wild-type (left) and *AtFPF1-4C* (right) plants grown under SD conditions.

(B) Paclobutrazol-treated wild-type (left) and *AtFPF1-4C* (right) plants grown under LD conditions.

(C) Four representative plants of a segregating F<sub>2</sub> population from a cross between *ga2-1* and *AtFPF1-8L* 5 weeks after sowing. The genotype of the plants from left to right is *ga2-1*, *ga2-1* expressing the *FPF1* transgene, WT, and *AtFPF1-8L*.

(D) Four representative plants of a segregating F<sub>2</sub> population from a cross between *ga4-1* and *AtFPF1-8L* 5 weeks after sowing. The genotype of the plants from left to right is *ga4-1*, *ga4-1* expressing the *FPF1* transgene, WT, and *AtFPF1-8L*.

(E) *ga2-1* mutant without (left) and with (right) the transgene 2 weeks later than the plants shown in (C).

(F) *ga4-1* mutant without (left) and with (right) the transgene 2 weeks later than the plants shown in (D).

than do control plants under noninductive conditions. Since then, it has been shown for several other species that GA treatment can overcome the vernalization or LD requirement for flower induction in rosette plants but does not cause flowering in SD plants (Zeevaert, 1983). Talon et al. (1991) and Zeevaert and Gage (1993) found that GA synthesis is enhanced under LD conditions in spinach, which may be due in part to the upregulation of the key enzyme of GA synthesis, the GA 20-oxidase, under LD conditions (Wu et al., 1996). A downregulation of the GA 20-oxidase transcript level after application of GA<sub>3</sub>, which supports the view that GA biosynthesis may be regulated by a feedback suppression of expression of the GA 20-oxidase genes in Arabidopsis, was shown by Phillips et al. (1995). That GAs are an

absolute requirement in Arabidopsis for flowering under SD conditions was reported by Wilson et al. (1992) using GA mutants. Furthermore, it was shown that the *spy* mutant, which exhibits a constitutive GA response, and the *phyB* mutant, with an increased GA responsiveness, also flowered earlier (Jacobsen and Olszewski, 1993; Reed et al., 1996).

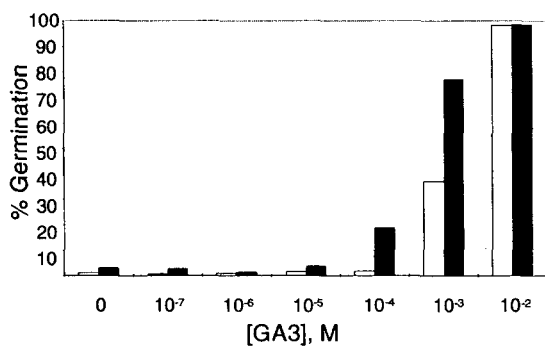
The initial observation that the *FPF1*-overexpressing plants resemble to a great extent wild-type plants treated with GA<sub>3</sub> led us to analyze in greater detail the possible involvement of *FPF1* in a GA-dependent signaling pathway. In a first series of experiments, the GA biosynthesis inhibitor paclobutrazol was used to downregulate GA synthesis (Rademacher, 1991). Under SD conditions in both wild-type and constitutively *FPF1*-expressing plants treated with pa-



paclobutrazol, flowering was not visible after 3 months of growth, and the plants showed the typical dwarf phenotype of plants deficient in GAs. Under LD conditions, however, the paclobutrazol treatment did not completely block flower formation. Inflorescence development in the wild-type plants was greatly retarded, whereas the transgenic plants formed an inflorescence. The failure of paclobutrazol treatment to block inflorescence development in the transgenic plants under LD conditions could be due to an upregulation of GA synthesis by the transgene during the first 10 days before paclobutrazol treatment or to an enhanced sensitivity to the normal level of GAs synthesized before paclobutrazol treatment.

Also, double mutants containing the *FPF1* transgene and either the *ga2-1* or *ga4-1* mutation showed a GA concentration-dependent phenotype. In the *ga2-1* and *AtFPF1-8L* cross, no early flowering occurred under SD conditions in the *ga2-1* mutant progeny expressing the transgene. In the leakier *ga4-1* mutant, expression of the *FPF1* transgene led to an earlier flowering and a stunted inflorescence under SD conditions, similar to that of paclobutrazol-treated transgenic plants under LD conditions, whereas the inflorescence development of the *ga4-1* mutant without the transgene was retarded. These genetic experiments again indicate that the function of the *FPF1* gene is dependent on the synthesis and existence of GAs but do not permit us to decide whether the synthesis of or the responsiveness to GAs is altered in the transgenic plants.

GA measurements with combined gas chromatography and mass spectrometry have shown that 2-week-old seedlings of constitutively *FPF1*-expressing plants contain only slightly higher amounts of  $GA_4$  and  $GA_{20}$  than do wild-type seedlings under both LD and SD conditions, which are



**Figure 8.** Effect of *FPF1* Overexpression on Germination.

Seeds from transgenic and wild-type plants were allowed to germinate on agar plates with medium containing 37 mg/L paclobutrazol supplemented with the indicated amounts of  $GA_3$ . Because seed development is very slow in the presence of paclobutrazol, germination was assayed 10 days after sowing. The germination of seeds from wild-type plants is shown by open bars and that of transgenic plants by solid bars.

much below the level that would be required for the development of the phenotype of the transgenic plants overexpressing *FPF1* (S. Melzer and P. Hedden, unpublished results). Although we do not know if this is due to the seedlings being at different physiological ages, because of the shortened life cycle of the transgenic plants, the slight increase of GAs could also be due to a feedback mechanism in the GA biosynthesis pathway. In case of an enhanced responsiveness to GAs, more GAs would be used by the transgenic plants by decreasing the pool of free GAs, which could be compensated by an additional GA biosynthesis.

We have observed several phenotypic alterations in the *FPF1*-overexpressing plants at places where we have observed no expression in the wild-type plants. In these tissues, the overexpression of *FPF1* could mimic the activity of the second, closely related gene. By using one of these pleiotropic effects in the *FPF1*-overexpressing plants, namely, the early germination of transgenic seeds, we could clearly show that seeds of transgenic plants in the presence of inhibiting paclobutrazol concentrations germinated on lower  $GA_3$  concentrations than did those of wild-type plants. This observation would be in accordance with a higher responsiveness of the transgenic plants to GAs. The fact that transgenic plants sprayed with  $GA_3$  flowered earlier than did either wild-type plants treated with saturating amounts of  $GA_3$  or untreated transgenic plants also points to an altered responsiveness to GAs. In this case, the endogenous GA content would be limiting in the untreated transgenic plants to allow the full expression of the *FPF1* transgene activity. Furthermore, the *FPF1*-overexpressing plants phenocopy to a great extent the *phyB* mutant of Arabidopsis, which shows an enhanced responsiveness to GAs (Reed et al., 1996). Therefore, we propose that the expression of *FPF1* in the apical meristem upon the photoperiodic induction of flowering of mustard and Arabidopsis leads to an enhanced responsiveness to GAs and a tissue-specific activation of a GA signaling pathway in the apical meristem, which enhances the initiation of the flowering process.

Although treatment with  $GA_3$  leads to earlier flowering of Arabidopsis, this was not shown for its close relative mustard (reviewed in Bernier, 1969). Because mustard is not a rosette plant, GAs probably are not limiting, and further application of GAs will not shorten the vegetative phase, as was shown for other nonrosette plants (Metzger, 1995). However, a better sensing of the available GAs by the activation of a tissue-specific pathway that increases the responsiveness to GAs upon photoperiodic induction of flowering could play an important role in establishing floral development also in those plants that do not react to GA treatment.

The expression of *FPF1* was also detected in floral meristems and in the anlagen of stamens, indicating that the *FPF1* function may also be required in these organs. The activity of *FPF1* in floral meristems would be in line with the observation that a GA signaling pathway is involved in determining floral meristem identity (Okamoto et al., 1996), and the expression of *FPF1* in the anlagen of stamens is in accordance

with the finding that GAs are essential for anther development (Jacobsen and Olszewski, 1991).

Although the exact function of the *FPF1* gene product remains to be elucidated, it seems to affect the transition to flowering by modulating a GA signaling pathway in the apical meristem. Because plants that constitutively express *FPF1* do not differ from wild-type plants in their development of reproductive organs and in their seed set, the constitutive expressed *FPF1* gene may confer a new and useful trait on crop plants for flowering time control.

## METHODS

### Growth of Plants

Mustard (*Sinapis alba*) plants were grown on soil in phytotrons at 20°C under fluorescent bulbs emitting a light intensity of 150 to 200  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ . After 56 days under 8-hr short-day (SD) conditions, flowering was induced with 16-hr photoperiods. *Arabidopsis thaliana* plants of the Columbia (Col) and Landsberg *erecta* (Ler) ecotypes were grown under either 8-hr SD or 16-hr long-day (LD) conditions at 20°C with a light intensity of 150  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ .

### cDNA Cloning

A cDNA library from apices of plants that were induced to flowering by 7 long days was established in  $\lambda\text{gt}10$  from RNA that was prepared from dissected apical meristems, as described by Menzel et al. (1996). Five hundred thousand phages of the 7th-day-stage library were screened with the radiolabeled mustard *flowering promoting factor1* (*FPF1*) cDNA fragment. The hybridization was performed at 62°C in 6  $\times$  SSC (1  $\times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5  $\times$  Denhardt's solution (0.1% Ficoll, 0.1% PVP, and 0.1% BSA), 0.1% SDS, and 100  $\mu\text{g}/\text{mL}$  herring sperm DNA. High-stringency washes were done three times at 62°C for 10 min. The homologous *Arabidopsis* cDNA was identified in a cDNA library from young inflorescences (prepared by Weigel et al. [1992] and provided by the *Arabidopsis* Biological Resource Center, Ohio State University, Columbus) and screened with the mustard *FPF1* cDNA, as described above, under low-stringency conditions. After two rounds of rescreening, phage inserts were subcloned into pBluescript II SK+ (Stratagene), and both strands were sequenced with the T7 DNA polymerase by the dideoxy chain termination method. Sequencing solutions and buffers were prepared according to U.S. Biochemical. Sequencing primers were synthesized on DNA synthesizer (Applied Biosystems, Foster City, CA). DNA sequences were aligned and compared with the Genetics Computer Group (Madison, WI) sequence analysis software package (Devereux et al., 1984). All DNA manipulations were performed as described by Ausubel et al. (1989). Mapping of the *FPF1* gene of *Arabidopsis* was performed according to Lister and Dean (1993). The mapped position was compared with the data in the *Arabidopsis* database (<http://genome-www.stanford.edu/Arabidopsis/>).

### RNA Gel Blot Analysis

For gel blots, RNA was extracted from apical buds of vegetative and induced mustard plants, which were freed of leaf primordia with a

pair of tweezers and immediately frozen in liquid nitrogen. The RNA from all examined tissues was isolated with guanidinium isothiocyanate, as described by Melzer et al. (1990). Poly(A)<sup>+</sup> RNA was isolated by small-scale oligo(dT) chromatography, as described for standard methods (Ausubel et al., 1989). Two micrograms of mRNA from leaves and apical buds from vegetative and induced mustard plants were separated on a 1% agarose gel containing 1.5% formaldehyde and transferred onto reinforced nitrocellulose membranes (Schleicher & Schuell). Hybridizations were performed overnight at 42°C in a solution containing 50% formamide, 5  $\times$  SSPE (1  $\times$  SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5  $\times$  Denhardt's solution, 0.1% SDS, and 100  $\mu\text{g}/\text{mL}$  herring sperm DNA. The nitrocellulose filters were washed first in 1  $\times$  SSC with 0.1% SDS at 68°C for 10 min and subsequently twice in 0.1  $\times$  SSC and 0.1% SDS for 10 min at 68°C. The membranes were exposed to Kodak XAR film for 7 days.

### In Situ Hybridizations

Apical buds were fixed in 4% formaldehyde, 50% ethanol, and 5% acetic acid for 5 hr at 4°C. The fixed buds were dehydrated and embedded in paraffin by using standard methods. Longitudinal sections (10  $\mu\text{m}$ ) were cut with a rotary microtome, using metal knives. These sections were transferred onto poly-L-lysine-coated slides. <sup>35</sup>S-UTP-labeled antisense and sense riboprobes were prepared by using T3 or T7 RNA polymerase. The labeled transcripts were partially hydrolyzed to an average length of 150 bases. In situ hybridizations were performed essentially as described by Melzer et al. (1990). After the final washes, the dried slides were coated with NTB photoemulsion (Kodak) and exposed for 3 to 4 weeks. Developed sections were stained with toluidine blue, dehydrated with ethanol, transferred to xylene, and mounted with DePeX (Serva, Heidelberg, Germany). The sections were viewed through an Axiophot microscope (Carl Zeiss, Oberkochen, Germany), and photographs were taken on Ektachrome 64T film (Kodak) as double exposures from the bright field and with a red filter from the dark field. Pictures were processed with Photoshop (Adobe Systems, Mountain View, CA) and Designer (Micrografix, Richardson, TX) software.

### Constitutive Expression of *FPF1*

The protein-coding regions of the *FPF1* cDNAs from mustard and *Arabidopsis* were amplified via polymerase chain reaction using primers that introduced restriction sites at the ends of the amplification products. The primer CRD (5'-TGCAGGATCCACCATGGCAGGCGTGTGGGTGTTTC-3') of the 5' end of the coding region contains an NcoI site at the ATG start codon and a BamHI site before the NcoI site. The 3' end primer CRU (5'-ATGCGGATCCTTAATTGAGTCTCGGAC-3') introduced a BamHI site after the stop codon. The polymerase chain reaction products were ligated via the BamHI sites into the plasmid vector pBluescript II SK+ and sequenced. The amplified coding regions were introduced via NcoI and BamHI into the vector pSH9 that contains a cauliflower mosaic virus 35S promoter with an  $\Omega$  element as a translational enhancer adjacent to the NcoI site and a translational terminator with a polyadenylation site adjacent to the BamHI site (Holtorf et al., 1995). From the recombinant vectors pSH9-*FPF1*, the expression cassette was ligated as a HindIII fragment into the bi-

nary plant transformation vector pBIN19 (Bevan, 1984) to create the recombinant plant transformation vectors pBIN19-*FPF1*.

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* C58C1 by a standard transformation procedure (Höfgen and Willmitzer, 1988). The Arabidopsis ecotype Col was transformed with the vacuum infiltration method (Bechtold et al., 1993). Plant seeds were harvested 4 to 5 weeks after infiltration, sterilized, and plated on germination medium containing 500 mg/L timenten to suppress agrobacterial growth and 50 mg/L kanamycin for selection of transformants. Transformants were transferred to soil, and seeds were harvested; homozygous lines were selected and used for further analysis. The leaf number was determined as described by Wilson et al. (1992).

### Gibberellin and Paclobutrazol Treatments

For the gibberellin A<sub>3</sub> (GA<sub>3</sub>) treatment, 12 wild-type or transgenic plants that had been transplanted into individual pots 14 days after sowing and grown under SD conditions were sprayed once a week either with water or with 100 μM GA<sub>3</sub>. The paclobutrazol treatment was started under both LD and SD conditions after the wild-type and transgenic plants were singled out in pots. The plants were watered once a week with a solution containing 37 mg/L paclobutrazol (Duchefa, Haarlem, The Netherlands), according to Jacobsen and Olszewski (1993). For germination assays, agar plates with Murashige and Skoog salts (Sigma) were supplemented with 37 mg/L paclobutrazol and with different amounts of GA<sub>3</sub>. Approximately 100 seeds were placed on each plate, and seedlings were analyzed 14 days after sowing.

### Genetic Analyses of Crosses with *ga* Mutants

Plants carrying the *FPF1* transgene in the *ga2-1* and *ga4-1* background were constructed by pollinating the mutant plants with pollen from *AtFPF1-1L* plants. In the F<sub>1</sub> progeny, plants with the typical elongated *FPF1* phenotype were selfed to obtain the F<sub>2</sub> populations segregating for the *FPF1* transgene and the recessive *ga2-1* or *ga4-1* mutation. Surface-sterilized F<sub>2</sub> seeds were germinated under SD conditions on agar plates supplemented with 10 μM GA<sub>3</sub> and transferred to plates without GA<sub>3</sub> after 5 days. Two weeks after sowing, the plants were transferred to soil and further cultivated under SD conditions. The genotype of the analyzed plants was confirmed in the F<sub>3</sub> by kanamycin selection (50 μg/mL) and analysis of the mutant phenotype.

### Scanning Electron Microscopy

Samples were mounted on special holders with two-sided tape and frozen in liquid nitrogen. The holder was mounted on a Gatan (Pleasanton, CA) stage and cryosputter coated with 5 nm of platinum at -140°C. Afterward, the Gatan stage with the frozen sample was transferred into an S-900 in-lens field emission scanning electron microscope (Hitachi, Prophysics, Uster, Switzerland). The sample was imaged at -140°C by using backscattered electron signals as described in Walther and Müller (1997). The signal was recorded digitally with a Digi Scan interface and Digital Micrograph software (Gatan). Because the scanning area was restricted to 380 × 380 μm, each picture is composed of five individual images.

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