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FPA, a Gene Involved in Floral Induction in Arabidopsis, Encodes a Protein Containing RNA-Recognition Motifs

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***FPA* is a gene that regulates flowering time in Arabidopsis via a pathway that is independent of daylength (the autonomous pathway). Mutations in *FPA* result in extremely delayed flowering. *FPA* was identified by means of positional cloning. The predicted FPA protein contains three RNA recognition motifs in the N-terminal region. *FPA* is expressed most strongly in developing tissues, similar to the expression of *FCA* and *LUMINIDEPENDENS*, two components of the autonomous pathway previously identified. Overexpression of *FPA* in Arabidopsis causes early flowering in noninductive short days and creates plants that exhibit a more day-neutral flowering behavior.**

INTRODUCTION

To accurately gauge when to flower, plants use environmental cues such as daylength and temperature as well as developmental (often called autonomous) controls to coordinate synchronous flower production (Vince-Prue, 1975). As a result of physiological and genetic studies in Arabidopsis, many loci that specifically regulate flowering time have been identified (reviewed in Simpson et al., 1999).

Mutations in genes that promote flowering cause a late-flowering phenotype. Such genes have been grouped into two classes based on details of their mutant phenotypes. Mutations that result in late flowering that is independent of daylength define one group of genes that promotes flowering through inductive long-day photoperiods (Koornneef et al., 1991). In effect, these mutations are "blind" to inductive photoperiods; therefore, late-flowering, photoperiod pathway mutants flower as if they were grown in noninductive, short-day conditions. A second class of mutations results in delayed flowering that is still responsive to daylength (Koornneef et al., 1991). These mutants flower late in long days but even later in short days; therefore, these mutants define genes that promote flowering independently of daylength. Accordingly, these mutations define genes in a pathway often designated as autonomous to indicate that it is distinct from the photoperiod pathway.

The late-flowering phenotype of the autonomous-pathway mutants can be abolished by cold treatment of sufficient duration to promote flowering (i.e., vernalization), whereas mutants in the photoperiod pathway are relatively unaffected by vernalization (Koornneef et al., 1991). A number of genes that promote flowering in both the photoperiod and autonomous pathways have been cloned, including *CONSTANS* (*CO*) (Putterill et al., 1995), *GIGANTEA* (*GI*) (Fowler et al., 1999), *LUMINIDEPENDENS* (*LD*) (Lee et al., 1994a), *FCA* (Macknight et al., 1997), *FT* (Kardailsky et al., 1999), *FHA*/*CRY2* (Guo et al., 1998), *FLAVIN BINDING*, *KELCH REPEAT*, *F BOX* (*FKF1*) (Nelson et al., 2000), *SOC1* (Lee et al., 2000; Samach et al., 2000), and *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999; Sheldon et al., 1999).

The autonomous regulation of flowering time has been the focus of much research due to naturally occurring variation in this pathway. The majority of Arabidopsis accessions may be considered as either spring or winter annuals. Spring annual varieties do not require vernalization to flower rapidly. Without previous vernalization, winter annual varieties flower months later than spring varieties. However, winter varieties flower as rapidly as spring varieties when given a vernalization treatment (Napp-Zinn, 1969).

The late-flowering behavior of winter varieties is due to the presence of dominant alleles of two genes, *FRIGIDA* (*FRI*) and *FLC* (Napp-Zinn, 1979; Burn et al., 1993; Lee et al., 1993, 1994b; Clarke and Dean, 1994; Koornneef et al., 1994). Overexpression of *FLC* is sufficient to delay flowering in the absence of *FRI* (Michaels and Amasino, 1999; Sheldon et al., 1999) and *FRI*-containing plants have no discernible phenotype in the absence of *FLC* (Michaels and Amasino, 2001). Therefore, it appears that *FRI* functions solely to upregulate *FLC*, which in turn delays flowering.

FLC/*FRI*–containing plants exhibit delayed flowering that

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is reversed completely by vernalization (Lee and Amasino, 1995), similar to that of the autonomous-pathway mutants *fca*, *fy*, *fve*, *ld*, *fld*, and *fpa.* The phenotypic similarity of *FRI*-containing plants and autonomous-pathway mutants is a reflection of the antagonistic roles of *FRI* versus the wild-type function of autonomous-pathway genes in *FLC* regulation. Whereas *FRI* acts to increase *FLC* RNA levels, the autonomous-pathway genes act to decrease *FLC*. Thus, *FLC* message levels are increased in autonomous-pathway mutants similar to *FRI*-containing lines (Michaels and Amasino, 1999; Sheldon et al., 1999). Furthermore, the late-flowering phenotype of mutations in autonomous-pathway genes is abolished by the loss of *FLC* function, indicating that autonomous-pathway genes, like *FRI*, act solely to regulate *FLC* (Michaels and Amasino, 2001).

It is interesting that many genes that both promote and inhibit flowering appear to have evolved to regulate one gene, *FLC*. To understand the regulation of *FLC* expression, it is necessary to determine the components of the autonomous pathway that regulate *FLC*. *FPA* is one such component, and it was chosen for further investigation.

In this article, we describe the identification of *FPA*. *FPA* encodes an RNA recognition domain–containing protein. We show that the expression pattern for *FPA* is similar to that of other genes identified previously in the autonomous pathway. Additionally, we demonstrate that overexpression of this autonomous-pathway gene causes precocious flowering in short days.

RESULTS

Molecular Identification of *FPA*

A screen for embryo-defective auxotrophic mutants that were deficient in the synthesis of vitamins and amino acids was performed in the Columbia accession of Arabidopsis (Patton et al., 1998). The arrested embryos from embryo-defective mutants were grown on enriched medium and screened for compounds that would rescue the embryo-defective phenotype; one mutant that could be rescued by treatment with supplemental biotin defined a new biotin biosynthetic locus, *BIO2* (Patton et al., 1998). To recover viable homozygous *bio2* mutant seed, heterozygotes were treated daily with an external application of supplemental biotin (see Methods), and these biotin-treated heterozygotes yielded progeny that segregated for extremely late flowering plants (Patton et al., 1998). The *bio2* lesion was found to be a deletion that removed the *BIO2* gene, and the lack of *BIO2* provided a polymerase chain reaction (PCR)–based assay to determine the *bio2* genotype. In a segregating population of $>$300 plants from a biotin-rescued heterozygote, all plants heterozygous or wild type at the *BIO2* locus flowered similarly to wild-type plants, whereas the *bio2* homozygous mutants were late flowering. Thus, the lesion that removed *BIO2* is tightly linked genetically to the mutation that caused the late-flowering phenotype.

BIO2 is located near the bottom of chromosome II (Patton et al., 1998). The late-flowering phenotype of *bio2* homozygotes rescued with biotin was at first thought to result from loss of several linked genes within the deletion because none of the known flowering loci had been mapped to that chromosomal region (Patton et al., 1998). When subsequent map updates indicated that *FPA* might be closer to *BIO2* than previously thought, complementation tests were performed with *fpa* to check for allelism. Because *bio2* homozygotes are not viable without supplemental biotin, *bio2* heterozygotes were crossed to *fpa-2* and the progeny were scored for late flowering. Approximately 50% of the F1 progeny displayed wild-type flowering (after producing $\sim$7 to 10 leaves), whereas the other half of the population flowered after producing 45 to 55 leaves, similar to *fpa*. This result is consistent with noncomplementation of a heterozygous recessive mutation crossed to a homozygous recessive mutation in the same locus. Thus, the *bio2* deletion also appeared to remove the known flowering locus *FPA*. The *fpa* allele linked to the *bio2* deletion is referred to as *fpa-5* in this article. Other mutant alleles (*fpa-2* and *fpa-3*) are described by Meier et al. (2001).

To determine the degree of linkage of *FPA* to *BIO2* and to evaluate the possibility that *fpa-5* and *bio2* were caused by the same deletion, we developed a screen for recombination between *fpa-5* and *bio2* based on the embryo-lethal phenotype of *bio2* homozygotes without the addition of supplemental biotin. Plants heterozygous for the *bio2* lesion were self-pollinated in the absence of biotin, and the subsequent generation was assayed for late flowering. The presence of late-flowering progeny that would not require supplemental biotin would indicate a recombination event between *bio2* and *fpa*. No late-flowering recombinants were found among $\sim$10,000 plants, indicating that the linkage was extremely close and that *FPA* probably resided in the same deletion that removed *BIO2*.

DNA gel blot analyses (see Methods) revealed that the *bio2* and *fpa-5* lesions were part of a 32-kb deletion of a region predicted to encode nine genes in addition to *BIO2* (summarized in Figure 1). An additional allele of *fpa* (*fpa-6*) was isolated from a T-DNA–based mutagenesis, but the *fpa-6* allele did not cosegregate with the T-DNA; therefore, the lesion in the *FPA* gene in *fpa-6* did not contain a T-DNA insertion. Characterization of the *fpa-6* allele revealed that there was a 2.5-kb deletion within the 32-kb region deleted in *fpa-5*; thus, *fpa-6* narrowed the interval containing *FPA* to two candidate genes. The deletion in *fpa-6* removed the 3′ end of a putative steroid reductase and the promoter and 5′ coding region of an RNA recognition motif (RRM)–containing protein (Figure 1). To determine which transcript encoded *FPA*, the steroid reductase and the RRM-containing protein were sequenced in two ethyl methanesulfonate–induced *fpa* alleles, *fpa-1* and *fpa-2*. Point mutations were

detected in both alleles in the gene that encodes the RRM-containing protein. The point mutation in *fpa-1* converts arginine 401 to a stop codon, and the mutation in *fpa-2* converts tryptophan 174 to a stop codon (Figure 1). Thus, *FPA* appears to be encoded by a putative RRM-containing protein.

Rescue of *fpa-6* with Clones of the RRM-Containing Protein

To verify that the RNA recognition motif–containing protein was *FPA*, the smallest region that rescued the late-flowering phenotype was determined by transforming small clones in the region into *fpa-6*. A library with an insert size of 9 to 20 kb was created from three overlapping bacterial artificial chromosomes (T1O24, T4I14, and T6P16) that contained the *FPA* region. Transformation of this library into *fpa-6* resulted in several wild-type-flowering T1 plants (data not shown). The inserts derived from the wild-type-flowering T1 transformants were amplified by PCR from genomic DNA using primers that flanked the insert site. The three smallest clones were chosen for sequencing. Nested primers were used to sequence the insert ends to define the boundaries of the clones. Diagnostic digests were performed on the clones to verify that the insert contained the entire region encompassed by the defined end sequence. The only complete gene product represented on the rescuing clones was the protein containing the putative RRM-containing protein (Figure 1). The smallest clone that was found to rescue the mutant contained ~2.5 kb of 5′ DNA and 1 kb of 3′ DNA of the putative RRM-containing protein (Figure 1).

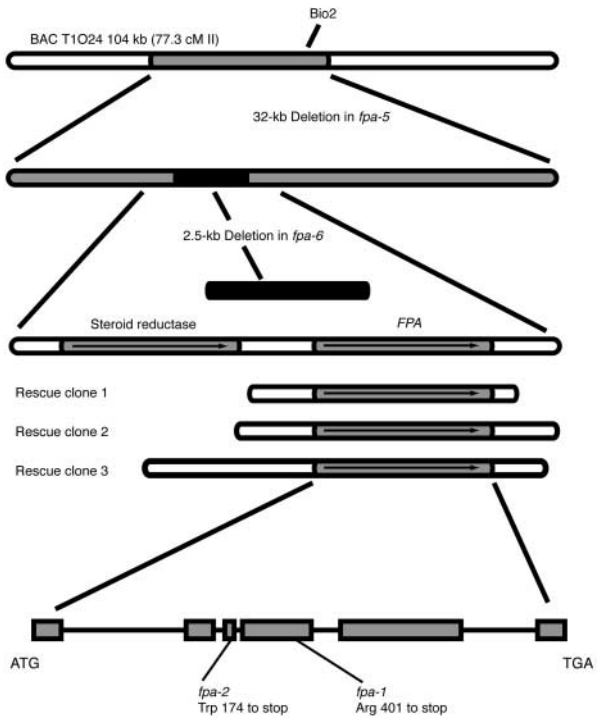

Figure 1. Cloning and Characterization of *FPA*.

Summary of the isolation of *FPA* based on deletion alleles (top). The deletion found in *fpa-5* approximately encompasses nucleotides 24,500 to 57,000 of bacterial artificial chromosome (BAC) T1O24. BAC T1O24 is located 77.3 cM (centimorgan) from the top of chromosome II. Schematic representations of genomic clones that rescued *fpa-6* mutants are shown in the middle. The genomic structure of *FPA* is shown at bottom; boxes designate exons and lines designate introns. Point mutations in *fpa-1* and *fpa-2* are shown.

FPA Contains RRMs

Isolation of an *FPA* cDNA revealed that the *FPA* gene is composed of six exons (Figure 1) that encode a 901–amino acid residue protein. The predicted FPA protein contains three RRMs (Birney et al., 1993), which indicates that FPA may function as an RNA binding protein. Alignment of the individual FPA RRMs to the closest matches of RRMs in other proteins shows that the majority of amino acid residues in each RRM of FPA are represented in the same positions of one or more of the RRMs of other proteins (Figure 2). Two small regions known as RNP-2 and RNP-1 consensus sequences define RRMs (Burd and Dreyfuss, 1994) and are shown boxed in Figure 2. The closest relative in the Arabidopsis genome is a predicted protein on chromosome IV that is 38% similar to FPA (CAB78307). The similarity between FPA and CAB78307 is distributed equally throughout the protein, and 15 introduced gaps are required to achieve this level of similarity (data not shown). There is a citrus expressed sequence tag (EST) C24205 that exhibits greater similarity to FPA than does CAB78307, although the EST spans only a portion of the gene.

Characterization of *fpa* Alleles

Alleles of *fpa* display different strengths of inhibition of floral induction. The *fpa-6* mutant in Wassilewskija (Ws) displays the strongest delay of flowering (Figure 3). The *fpa-6* deletion allele removes the entire 5′ regulatory region of *FPA* and the first exon of the coding region and is likely to be in a null allele. The two alleles in the Landsberg *erecta* (L*er*) accession (*fpa-1* and *fpa-2*) are not as late flowering as *fpa-6* in Ws (flowering behavior is presented in Tables 1 and 2), because the L*er FLC* allele is a partial suppressor of *fpa* mutants (Sanda and Amasino, 1996). Thus, a direct comparison of the *fpa* alleles cannot be made. However, *fpa-2* is clearly later flowering than *fpa-1*, indicating that *fpa-1* may not represent a null allele.

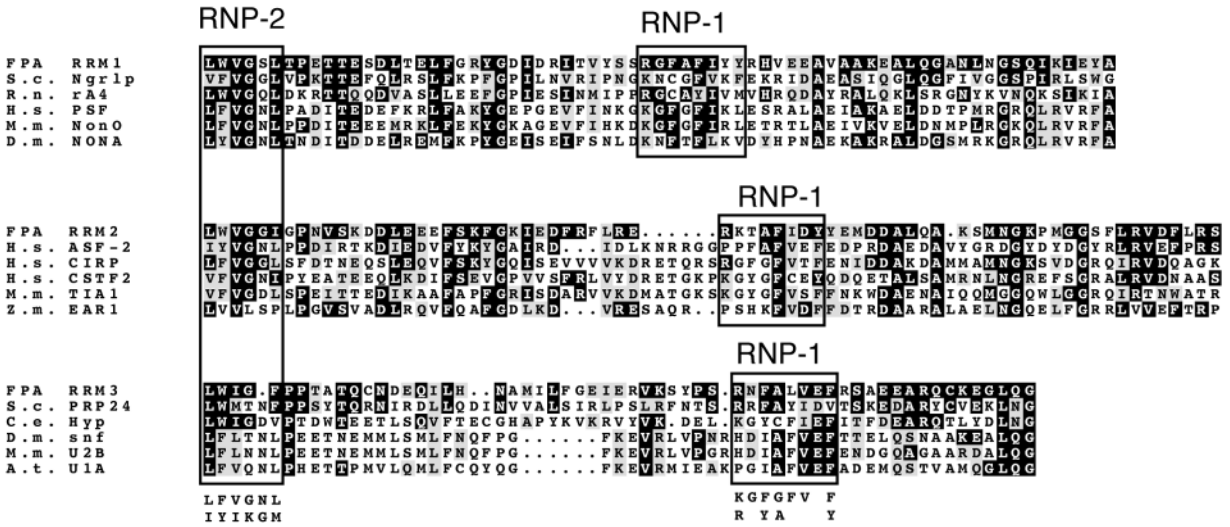

Figure 2. Amino Acid Sequence Alignment of FPA RRMs.

Three RRMs of FPA are shown aligned to the most similar RRMs in the databases. Boxed regions show RNP-2 and RNP-1 subdomains of RRM regions. Consensus regions for RNP domains are defined by Burd and Dreyfuss (1994). Black boxes denote amino acid residues identical to those in FPA, and gray boxes denote similar amino acid residues. FPA RRM1 is shown aligned to *Saccharomyces cerevisiae* Negative Growth Regulatory Protein (S.c. Ngr1p), *Rattus norvegicus* C-terminal domain binding protein (R.n. rA4), *Homo sapiens* PTB-associated Splicing Factor (H.s. PSF), *Mus musculus* octamer binding protein (M.m. NonO), and *Drosophila melanogaster* No-on-transient A (D.m. NONA). FPA RRM2 is shown aligned to *H. sapiens* alternative splicing factor (H.s. ASF-2), *H. sapiens* cold inducible RNA binding protein (H.s. CIRP), *H. sapiens* cleavage stimulation factor (H.s. CSTF2), *M. musculus* RNA binding protein (M.m. TIA1), and *Zea mays* EAR1 protein (Z.m. EAR1). FPA RRM3 is shown aligned to *S. cerevisiae* U6 snRNP protein (S.c. PRP24), *Caenorhabditis elegans* hypothetical protein T07F10.3 (C.e. Hyp), *D. melanogaster* splicing protein snf (D.m. snf), *M. musculus* snRNP (M.m U2B), and *Arabidopsis thaliana* snRNP-specific protein (A.t. U1A).

Overexpression of FPA Results in Early-Flowering in Short Days

To determine the effect of increased FPA activity, we designed a construct for overexpression in which the genomic coding region (start to stop codon) was joined with the constitutive cauliflower mosaic virus 35S promoter and the nopaline synthase terminator region. Plants transformed with this construct, however, displayed one of two phenotypes, either later or earlier flowering than wild-type plants.

The frequency with which the late-flowering class arose depended on the accession that was transformed. In the first transformed generation (T1), late-flowering phenotypes were seen at the highest frequencies in accession Columbia. For example, in one experiment, 82% of the plants (59 of 72) in the T1 generation in accession Columbia were >10 leaves later flowering than the wild type. In Ws, <10% of the T1 population was more than five leaves later flowering than wild-type plants. However, the severity of the late-flowering phenotype in Ws transformants increased in the next generation; the progeny of many of these slightly late flowering T1 plants became severely late flowering and produced at least 30 more leaves than wild-type Ws (data from representative lines are shown in Table 1). Furthermore, this more severe late-flowering phenotype segregated in a fully dominant manner (3:1 late:early flowering), and late flowering cosegregated with the transgene (data not shown).

Thus, many lines transformed with a construct designed for overexpression yielded late-flowering plants that were phenotypically similar to *fpa* mutants, and the late-flowering phenotype was dominant and cosegregated with the transgene. Furthermore, the late-flowering phenotype displayed from sense expression constructs was suppressed by vernalization, similar to that of *fpa* mutants (data not shown). The existence of dominant mutants that contain a construct designed to increase mRNA levels but that exhibit a phenotype similar to loss-of-function mutants is likely to be a result of gene silencing (Fire, 1999).

To identify transgenic lines in which the expression of this construct resulted in increased *FPA* activity, we introduced the overexpression construct into *fpa-2* mutants, which are in the L*er* background. Transgenic lines in which the late-flowering phenotype of *fpa-2* was rescued were identified (Table 2 and Figure 3). From 144 primary transformants, 55 of the plants displayed wild-type flowering when grown under long-day photoperiod conditions. Three representative

lines were chosen for further characterization. When these 35S::*FPA* plants were grown under short-day conditions, they flowered much more rapidly than the wild type (Table 2 and Figure 3). Thus, *FPA* overexpression results in plants that flower after producing a similar number of leaves in either inductive or noninductive photoperiods.

FPA Expression Pattern Is Similar to That of *FCA* and *LD* and Is Expressed in Nonflowering Plants

Because the *FPA* RNA could not be detected by RNA gel blotting, the spatial localization of *FPA* RNA was determined by reverse transcription–PCR of RNA samples isolated from various plant tissues (Figure 4). The expression of *FPA* was compared with that of *FCA* and *LD*, two other genes in the autonomous pathway. The expression patterns for the three genes are remarkably similar. *FPA* expression can be detected in most tissues, but it is found at the highest levels in the flower stem and meristematic regions of the plant.

To further explore the spatial and temporal expression pattern of FPA, we fused a 1.8-kb fragment of genomic sequence 5′ to the start codon of *FPA* to the β-glucuronidase (GUS) reporter gene and determined the staining pattern of plants containing this construct (Figure 5). *FPA* expression based on GUS activity is similar to the reverse transcription–based PCR data except for floral buds that appear to possess weak GUS activity. The high *FPA* expression seen in flowers in the reverse transcription–based PCR DNA gel blot may be due to the expression of *FPA* in the flower peduncle, a tissue that was included in the samples for reverse transcription–PCR. Overall, *FPA* is expressed throughout the plant life cycle, indicating that activation of *FPA* expression is not a binary switch that controls floral induction; rather, *FPA* may be available to constantly promote flowering during development. In addition, *FPA* is most highly expressed in young and actively growing tissues, and as the tissue matures the expression decreases. For example, the young third and fourth leaves in the 13-day postgermination plant possess GUS activity, whereas the third and fourth leaves in the more mature 19-day postgermination plant do not possess any detectable GUS activity (Figure 5).

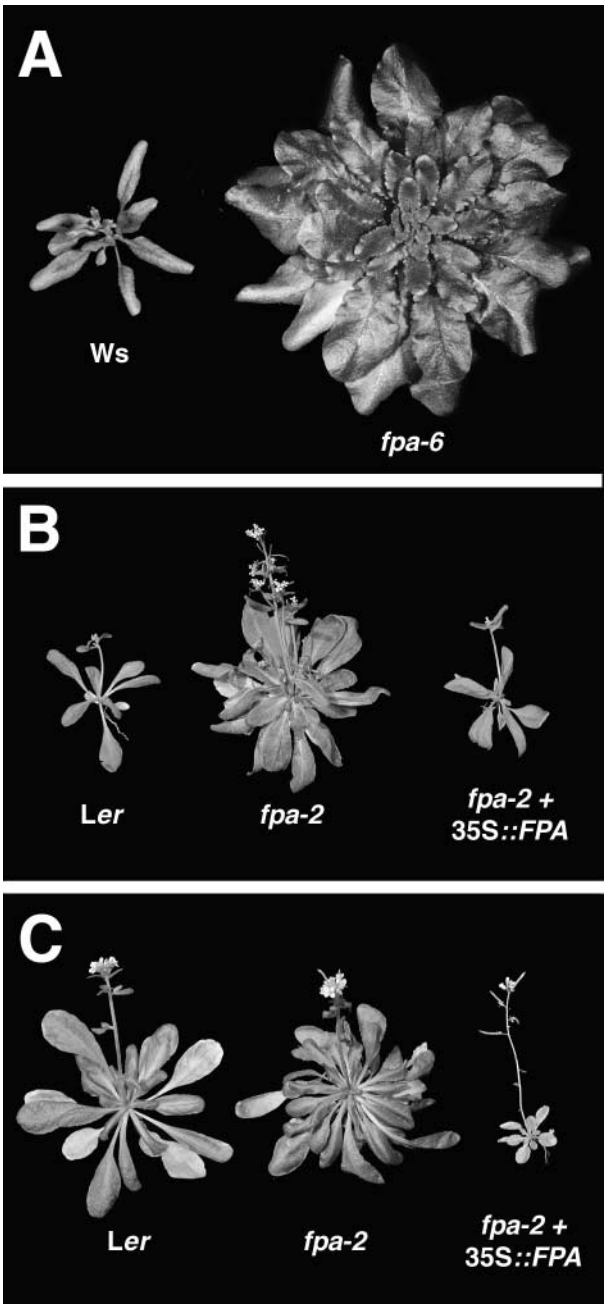

Figure 3. Flowering Phenotypes of Lines Used in this Study.

(A) Ws and *fpa-6* in Ws grown under long-day photoperiods.
(B) L*er*, *fpa-2* in L*er*, and *fpa-2* + 35S::*FPA* in L*er* grown under long-day photoperiods.
(C) L*er*, *fpa-2* in L*er*, and *fpa-2* + 35S::*FPA* in L*er* grown under short-day photoperiods.

DISCUSSION

Genetic and physiological analysis of late-flowering mutants in Arabidopsis has revealed two phenotypic classes. One class does not respond to daylength differences and vernalization treatments (photoperiod-pathway mutants), whereas the second group (autonomous-pathway mutants) responds to these environmental cues. Therefore, the autonomous pathway is thought to represent a developmental flowering program. In this study, we report the molecular identification

Table 1. Rosette Leaf Number at Flowering of *fpa-6* and *FPA* Cosuppression Lines

Genetic Background	Leaf Number at Flowering in Long Days[a]
Ws	7.5 ± 0.5[b]
Ws *fpa-6*	46.1 ± 2.0
Ws *FPA* cosuppression line 1	43.3 ± 3.6
Ws *FPA* cosuppression line 2	42.4 ± 4.1

[a] Leaf number was determined using only rosette leaves; cauline leaves were not counted.
[b] Ten plants were measured to determine flowering time data. Error is displayed as 1 SD.

and characterization of the autonomous-pathway component *FPA*.

FPA is predicted to be a 901–amino acid residue protein that contains three N-terminal RRMs. RRMs contain a degenerate consensus sequence in which no single position is conserved completely; rather, RRMs are composed of a similar framework of uncharged and hydrophobic amino acid residues. In addition to amino acid sequence variability, RRMs also are variable in length (Birney et al., 1993). The crystal structure of the RNA binding protein Sex-Lethal from Drosophila demonstrates that RRMs interact directly with single-stranded RNA and therefore are likely to mediate RNA binding specificity (Handa et al., 1999). Thus, this sequence and length variability may confer unique RNA sequence binding specificities.

FPA may function similarly to other proteins that contain RRMs. RNA binding proteins are involved in various aspects of RNA metabolism such as alternate splicing, stabilizing and destabilizing mRNAs, and regulation of translation initiation. However, the primary sequence of FPA does not provide any clues as to the specific aspect of RNA metabolism that FPA might affect. Outside the RRM regions, FPA does not exhibit significant similarity with any proteins of known function. FCA is another RNA binding protein in the autonomous pathway (Macknight et al., 1997). However, there is only weak similarity between FPA and FCA; in fact, the RRM regions of several RNA binding proteins from animals are more similar to FPA than are the RRMs of FCA. Also, outside of the RRMs, there is no obvious similarity between FPA and FCA.

Attempts to ascribe a function to *FPA* may be aided by previous work that has shown that *FLC* is the predominant negative regulator of the autonomous-flowering pathway. *FLC* appears to be the convergence point of the inhibition of flowering that is mediated by dominant alleles of the *FRI* locus and the promotion of flowering by the autonomous-pathway genes *FVE*, *LD*, *FCA*, and *FPA* (Michaels and Amasino, 1999; Sheldon et al., 1999). The *FRI*-mediated inhibition of flowering is due to increased levels of *FLC* mRNA. The converse is true of autonomous-pathway genes; these genes promote flowering by acting to decrease the levels of *FLC* mRNA. Therefore, *FLC* message levels act as an internal rheostat for flowering time that is positively regulated by *FRI* and negatively regulated by autonomous-pathway genes, such as *FPA* (Michaels and Amasino, 2001).

There are many mechanisms by which an RNA binding protein such as FPA might decrease *FLC* mRNA levels. For example, FPA may interact directly with *FLC* mRNA to affect splicing or stability. Alternatively, FPA may regulate other autonomous-pathway genes that in turn regulate *FLC* gene expression. FPA is the second example of an RRM-containing RNA binding protein in the autonomous pathway. FCA is the other RRM-containing RNA binding protein (Macknight et al., 1997), and LD encodes a homeobox-containing protein that might function as an RNA binding protein (Aukerman et al., 1999). Like *FPA*, both *FCA* and *LD* negatively regulate *FLC* message levels. Because of the preponderance of RNA binding proteins in the autonomous pathway, it is tempting to speculate that the autonomous pathway regulates *FLC* message levels post-transcriptionally.

It is possible that FRI may interact directly with the autonomous pathway. The late-flowering phenotype of plants containing dominant alleles of *FRI* or autonomous-pathway

Table 2. Rosette Leaf Number at Flowering of *fpa* Alleles and Overexpression Lines of *FPA*

Genetic Background	Leaf Number at Flowering in Long Days[a]	Leaf Number at Flowering in Short Days[a]
L*er*	7.2 ± 0.6[b]	23.9 ± 1.3
L*er fpa-1*	14.7 ± 1.0	Not determined
L*er fpa-2*	17.8 ± 1.4	49.2 ± 2.2
L*er fpa-2* + 35S::*FPA* line 1	8.0 ± 0.7	10.3 ± 1.5
L*er fpa-2* + 35S::*FPA* line 2	8.1 ± 0.6	10.5 ± 1.4
L*er fpa-2* + 35S::*FPA* line 3	8.3 ± 1.1	10.2 ± 1.1

[a] Leaf number was determined using only rosette leaves; cauline leaves were not counted.
[b] Ten plants were measured to determine flowering time data. Error is displayed as 1 SD.

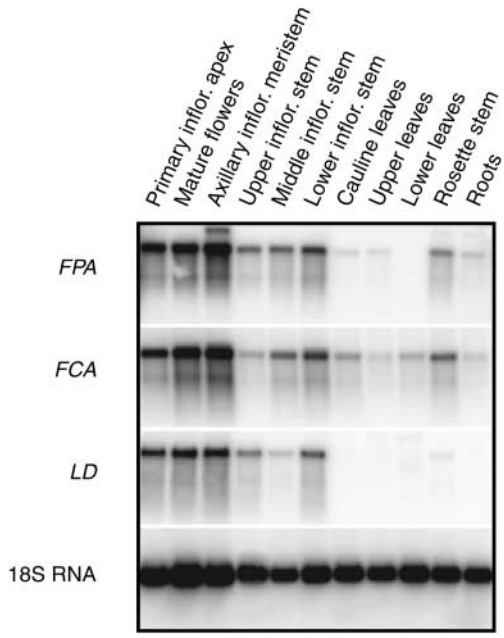

Figure 4. Reverse Transcription–PCR Analysis of *FPA*, *FCA*, and *LD* Expression Patterns.

FPA, *FCA*, and *LD* expression patterns were determined by reverse transcription–PCR. Twenty-five cycles of PCR amplification were used for each sample except the control 18S RNA product, which was detected at 17 cycles. Tissue for these experiments was obtained from the Ws accession at 22 days after germination. The inflorescence height at the time of tissue harvest was between 16 and 19 cm. The primary inflorescence (inflor.) apex consisted of the uppermost 3 mm of the inflorescence and contained immature floral buds. Flowers were taken on the day of anthesis. Axillary inflorescence meristem tissue was isolated with 2 mm of stem on either side of the meristem. Upper, middle, and lower inflorescence stem are defined as internode stem sections of the primary inflorescence. The lower stem refers to the first internode from the rosette followed by middle stem and upper stem, respectively. Stem sections did not contain shoot meristems. Cauline leaves are the oldest two leaves of the inflorescence. Upper leaves are defined as the youngest two leaves of the rosette, whereas the lower leaves are the oldest two rosette leaves. Rosette stem was obtained by removing roots, leaves, and the inflorescence from the rosette. Root tissue was isolated from plants grown on sterile medium (see Methods).

mutations are identical; in both cases, *FLC* mRNA levels are high and vernalization causes early flowering and a decrease of *FLC* mRNA levels (Michaels and Amasino, 2000). Because *FRI* is epistatic to the autonomous pathway (i.e., *FRI* causes late flowering in a background that is wild type for autonomous-pathway genes such as *FPA*), *FRI* could function by blocking the ability of *FPA* or other autonomous-pathway genes to downregulate *FLC* mRNA levels.

Although *FPA* clearly is a negative regulator of *FLC*, the fact that overexpression of *FPA* in short days results in early flowering indicates that *FPA* may have other functions. Null mutants of *FLC* are only slightly earlier flowering than wild-type plants in short days (Michaels and Amasino, 2001). Overexpression of *FPA* results in much earlier flowering than does loss of *FLC* gene function. Thus, one possibility is that *FPA* is a negative regulator of *FLC* and other flowering repressors and that *FPA* overexpression negatively regulates a collection of flowering repressors. It is also possible that 35S::*FPA* may interact positively with the photoperiod pathway, or with genes downstream of the photoperiod pathway, to produce early-flowering plants in short days. Overexpression of either *FT* or *CO*, components of the photoperiod pathway, produce essentially day-neutral plants that flower as early in short days as they do in long days (Kardailsky et al., 1999; Samach et al., 2000). Additionally, overexpression of *SOC1*, a MADS-box transcription factor that acts downstream of *CO*, also results in day-neutral early flowering (Lee et al., 2000; Samach et al., 2000). Because *FPA* overexpression lines flower with a similar number of leaves regardless of photoperiod conditions, it is possible that *FPA* may interact with similar downstream targets of photoperiod pathway genes. It must be noted that ectopic overexpression of

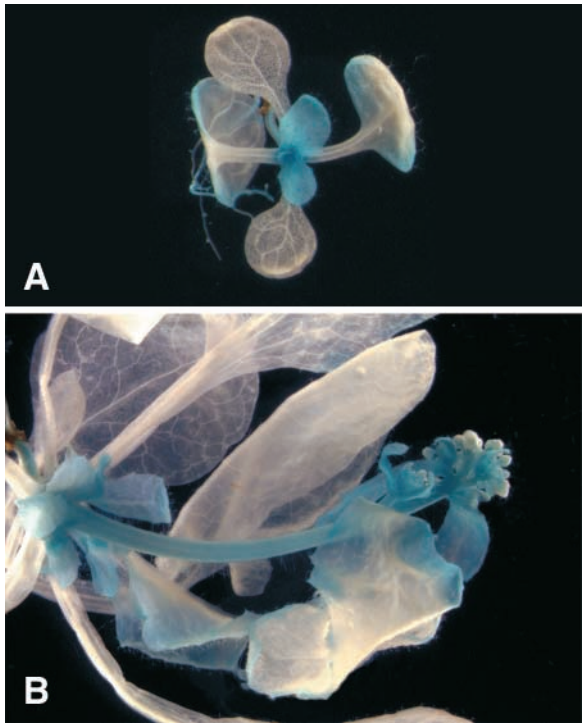

Figure 5. *FPA*::GUS Fusion Analysis of *FPA* Expression

(A) *FPA*::GUS expression in Ws before flowering, 13 days after germination.

(B) *FPA*::GUS expression in Ws during flowering (20-mm inflorescence), 19 days after germination.

FPA may affect genes that are not normally affected by the wild-type *FPA* locus.

Recent genetic analyses also indicate that *FPA* may have a broad role in plant development and flowering time regulation. Analysis of all possible double mutant combinations of the late-flowering mutants revealed differences between *fpa* and other autonomous-pathway mutants (Koornneef et al., 1998). Most notably, mutants in *fpa* displayed the most extreme late-flowering phenotype of the autonomous-pathway mutants when combined with the photoperiod-pathway mutants *fe* and *ft*, indicating that *FPA* may promote flowering through multiple pathways. Additionally, the double mutant between autonomous-pathway mutants *fpa-1* and *fy* was not attainable, presumably because of early lethality, indicating that *FPA* and *FY* are vital, but redundant, for a function in addition to flowering (Koornneef et al., 1998). Further investigation will be required to reveal the molecular basis of the role of *FPA* in flowering time regulation and possibly in other aspects of plant development.

METHODS

Biotin-Mediated Rescue of the Embryo-Defective Phenotype of *bio2* Homozygotes

Arabidopsis thaliana plants heterozygous for the *bio2* lesion were sprayed twice daily with a 1-mM aqueous biotin (Sigma, St. Louis, MO) solution until seed had matured. With daily biotin supplementation, homozygous *bio2* mutants formed ~65 leaves and then initiated flowering; however, even with continued biotin supplementation, *bio2* mutants did not produce viable seed.

Determination of Deletion Size of Lesion in *bio2*

Bacterial artificial chromosome (BAC) T1O24 was used a probe for hybridization to genomic DNA isolated from the *bio2* homozygous mutant to determine the size of the deletion. Briefly, bio2 and wild-type genomic DNA were digested with EcoRI and run on a 1% agarose gel, transferred to a nylon membrane, and probed with labeled T1O24 DNA. The radioactivity was detected using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA), and the *bio2* lane was compared with the wild-type lane for the absence of bands (data not shown).

Generation of Rescuing Clone Library

BACs T1O24, T4I14, and T6P16 were partially digested with Sau3AI and ligated into the BamHI site of 3300s. A total of 3000 clones containing inserts ranging in size from 6 to 20 kb were isolated and transformed into the *Agrobacterium tumefaciens* strain ABI. *fpa-6* mutant plants were then transformed with the entire library. Early-flowering plants were identified among the first-generation transformants. The inserts were amplified from the transformants with Takara LA Taq polymerase (Panvera, Madison, WI). Polymerase chain reaction (PCR) conditions were an initial denaturation of 3 min at 95°C, followed by 35 cycles of 20 sec at 98°C and 15 min at 68°C and then 10 min at 72°C (final extension). The primers used for the amplification step were 5′-ttgcatgcctgcaggtcgac-3′ and 5′-tacgaattcgagctcggtac-3′. Sequencing primers were 5′-tgcttccggctcgtatgttg-3′ and 5′-tatattactaattaattggggacc-3′.

Molecular Markers for Genotypic Analysis of *fpa-1* and *fpa-2* Alleles

Cleaved amplified polymorphic sequences were designed for the alleles of *fpa*. *fpa-1* can be identified using primers 5′-cacaaggtacgaggcgccctatga-3′ and 5′-ccactgatccatctcttcctggaa-3′. These primers result in a 100-bp fragment that is cleaved with AclI (New England Biolabs, Beverly, MA) to yield 76- and 24-nucleotide fragments in the wild type but is not cleaved in *fpa-1*. *fpa-2* can be identified using 5′-ttgtgttatcttcaggaacac-3′ and 5′-ctagtaacaagagacatactt-3′. These primers give a 100-bp PCR product that in *fpa-2* cleaves at 22 bp with BfaI (New England Biolabs) but that is not cleaved in the wild type.

Generation of Overexpression Construct of *FPA*

The overexpression construct of *FPA* was created by PCR amplification of *FPA* from the start codon to the stop codon from genomic DNA. The primers used to generate overexpression of *FPA* were 5′-aaa**ggatcc**atggcgttatctatgaagccattcagagcc-3′ and 5′-aaa**gagctc**tcaaggcccctgtccagccggagtacc-3′ (restriction sites shown in boldface, sequence corresponding to *FPA* is underlined), which generated a 4593-bp *FPA* fragment that was digested with BamHI and SacI and ligated into the BamHI and SacI sites of pRAM1. pRAM1 was produced by ligating the HindIII-EcoRI fragment of pBI121 containing the 35S promoter and nopaline synthase terminator sequences into the HindIII and EcoRI sites of pPZP211 (Hajdukiewicz et al., 1994). The construct was transferred into the Agrobacterium strain ABI and transformed into Arabidopsis through floral dipping (Clough and Bent, 1998).

mRNA Detection by Reverse Transcription–Mediated PCR

RNA was prepared from tissue frozen in liquid nitrogen. RNA was isolated with TRI reagent (Sigma) according to instructions for subsequent reverse transcription reactions. Five micrograms of total RNA was annealed to 500 ng of random decamer oligonucleotides. Superscript II reverse transcriptase (Gibco Life Technologies, Gaithersburg, MD) was used to generate cDNA. Takara ExTaq (Panvera) was used for PCR amplification of cDNA for desired products. PCR cycle conditions were as follows: 26 cycles of 15 sec at 95°C, 30 sec at 55°C, and 2 min at 72°C. Primers were used to detect full-length message of *LD*, *FPA*, and *FCA*-γ. Primers used for PCR amplification of *FPA* were 5′-atggcgttatctatgaagccattcagagcc-3′ and 5′-tcaaggcccctgtccagccggagtacc-3′; primers used for *LD* were 5′-ctcatgtactggctattcccttgg-3′ and 5′-tcgatcagctccaagatgtcgtcg-3′; primers used for *FCA* were 5′-atgaatggtcccccagatagagtag-3′ and 5′-tcatcaagctttattcttccacatgagttc-3′; primers used for 18S rRNA were obtained from Ambion (Austin, TX; catalog number 1718). Amplified DNA was transferred to nylon membranes (Gelman, Ann Arbor, MI) using standard protocols (Sambrook et al., 1989). DNA probes for hybridization were labeled with ^{32}P using the Prime-a-Gene labeling system, as described by the manufacturer (Promega, Madison, WI).

Histochemical β-Glucuronidase Assays

Whole plants, grown under long-day photoperiods, were fixed in 90% acetone for 1 hr at 4°C and were washed three times for 30 min in 50 mM $NaPO_4$, pH 7.0. Staining for β-glucuronidase (GUS) activity was performed by incubating plants in 50 mM $NaPO_4$ with 0.5% Triton X-100, 0.5 mM X-gluc (Research Organics Inc., Cleveland, OH), 0.5 mM $K_3(Fe[CN]_6)$, and 0.5 mM $K_4(Fe[CN]_6)$ for 12 hr. The plants were cleared with three 12-hr washes of 70% ethanol. The construct to determine the GUS expression pattern was generated by PCR amplification of the FPA 5′ regulatory region using the primers 5′-aaa**ctgcag**tgagaagtctgatgacacaatcattcaatc-3′ and 5′-aaa**ggatcc**cccatcgggattgtttcaattgacgatcctatgg-3′; the boldface type indicates restriction sites used for cloning. The resulting amplification product was digested with PstI and BamHI and ligated to a PstI-BamHI–digested pPZPGUS vector (HindIII to EcoRI fragment of pBI101.2 in pPZP211).

Plant Growth Conditions

Plants were grown under fluorescent light (100 μE m^{-2} sec^{-1}; cool-white Sylvania [Danvers, MA]) at 24 ± 1°C. Plants were fertilized with 2 g/L Dyna-Grow 7-9-5 fertilizer (Dyna-Grow Corp., San Pablo, CA) at 2-week intervals. Daylengths were 8 hr of light and 16 hr of dark for short-day and 16 hr of light and 8 hr of dark for long-day conditions. Plants grown in sterile media for the isolation of root tissue were grown on 5.5% Agar plates containing 2 g/L Dyna-Grow 7-9-5 fertilizer and 0.5 g MES (Sigma) and pH adjusted to 5.7. Light conditions for plants grown on sterile media were the same as those used for soil-grown plants.

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