GAMYB-like Genes, Flowering, and Gibberellin Signaling in Arabidopsis[1]

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We have identified three Arabidopsis genes with GAMYB-like activity, AtMYB33, AtMYB65, and AtMYB101, which can substitute for barley (Hordeum vulgare) GAMYB in transactivating the barley α-amylase promoter. We have investigated the relationships between gibberellins (GAs), these GAMYB-like genes, and petiole elongation and flowering of Arabidopsis. Within 1 to 2 d of transferring plants from short- to long-day photoperiods, growth rate and erectness of petioles increased, and there were morphological changes at the shoot apex associated with the transition to flowering. These responses were accompanied by accumulation of GAs in the petioles (GA_1 by 11-fold and GA_4 by 3-fold), and an increase in expression of AtMYB33 at the shoot apex. Inhibition of GA biosynthesis using paclobutrazol blocked the petiole elongation induced by long days. Causality was suggested by the finding that, with GA treatment, plants flowered in short days, AtMYB33 expression increased at the shoot apex, and the petioles elongated and grew erect. That AtMYB33 may mediate a GA signaling role in flowering was supported by its ability to bind to a specific 8-bp sequence in the promoter of the floral meristem-identity gene, LEAFY, this same sequence being important in the GA response of the LEAFY promoter. One or more of these AtMYB genes may also play a role in the root tip during germination and, later, in stem tissue. These findings extend our earlier studies of GA signaling in the Gramineae to include a dicot species, Arabidopsis, and indicate that GAMYB-like genes may mediate GA signaling in growth and flowering responses.

Gibberellins (GAs) regulate many aspects of plant growth and development. In the seed and seedling these include the production of hydrolytic enzymes, germination, and growth. In the adult plant, GAs are important in leaf and stem elongation, flowering, anther development, and fruit set (Pharis and King, 1985).

Two classes of mutants have contributed much to an understanding of GA action (Thornton et al., 1999). One class includes dwarf mutants that are defective in GA biosynthesis. The other class includes response mutants such as Arabidopsis spindly (spy), GA-insensitive (gai), repressor of GA1–3 (rga), and the rice (Oryza sativa) d1 mutant. Many of the genes defined by these mutants have been cloned, but their molecular role in GA signaling is not yet fully understood (Jacobsen et al., 1996; Peng et al., 1997; Silverstone et al., 1998; Ashikari et al., 1999).

An alternative approach to understanding GA signal transduction has involved functional studies, particularly with aleurone cells of cereals. These studies have identified a number of early GA signaling steps that precede expression of hydrolytic enzymes such as α-amylase. These steps involve heterotrimeric G-proteins (Jones et al., 1998; Ueguchi-Tanaka et al., 2000) and cGMP (Penson et al., 1996), which may in turn control the barley (Hordeum vulgare) HvGAMYB gene, whose expression is induced by GAs (Gubler et al., 1995).

HvGAMYB encodes a transcriptional activator that binds specifically to a GA-response element in an α-amylase promoter (Gubler et al., 1995). Constitutive expression of HvGAMYB mimics the effects of GA application and is sufficient to activate the α-amylase promoter and the promoters of other GA-regulated genes in aleurone tissue (Cercos et al.,

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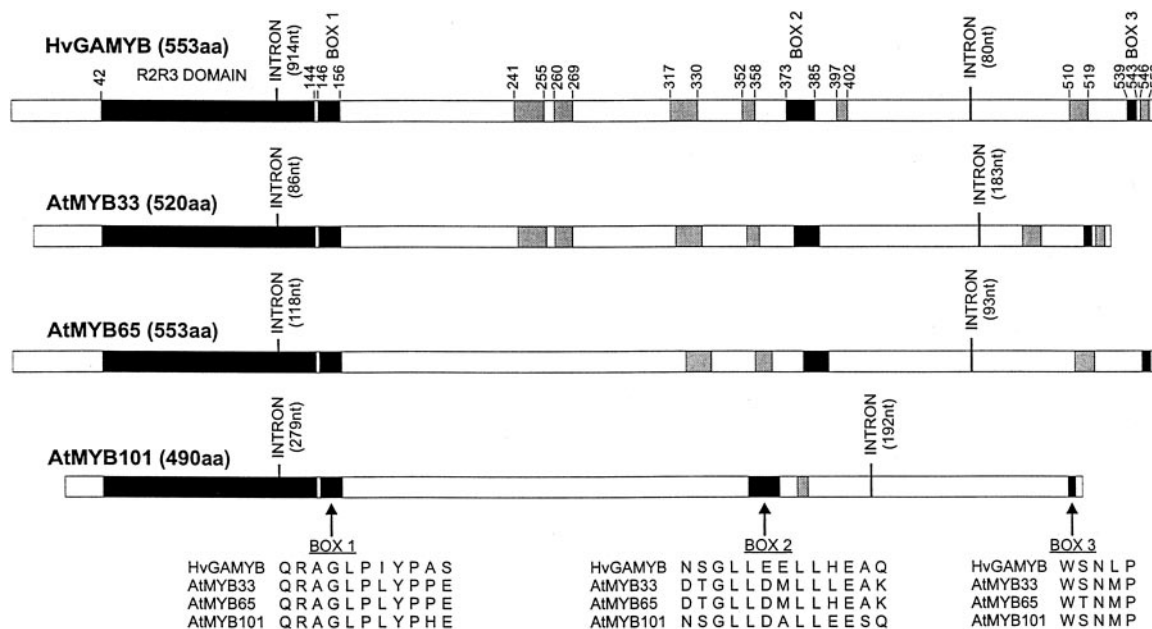
Figure 1. Comparison of the structure of the barley GAMYB protein (HvGAMYB) and the three Arabidopsis GAMYB-like proteins (AtMYB33, AtMYB65, and AtMYB101). Regions of similarity between all four proteins are shown in black, with the amino acid sequences of Boxes 1, 2 and 3 shown below. Regions of similarity between HvGAMYB and at least one Arabidopsis MYB are shown in gray. Similarity is defined as a continuous stretch of at least five amino acids with greater than or equal to 70% identity, or a continuous stretch of at least nine amino acids with greater than or equal to 50% identity. Amino acid numbers for the regions of similarity are shown for HvGAMYB. The location of the two introns present in the coding region of the genes is shown.

1999; Gubler et al., 1999). However, GAMYB involvement in the response to GAs may not be restricted to aleurone. For example, during long day (LD)-induced flowering of the grass *Lolium temulentum*, GAMYB expression increases at the shoot apex (Gocal et al., 1999), shoot apex GA content increases at this time (King et al., 2001), and applied GA application mimics the effects of LD exposure on floral induction (Evans, 1964; Evans et al., 1990; King et al., 2001). Thus, we have proposed that GA, acting via GAMYB, activates genes that are responsible for floral initiation/development.

A potential target for GAMYB in transcriptional regulation of flowering is the *LEAFY* gene. *LFY* is a potent inducer of flowering in dicots, including Arabidopsis (Weigel and Nilsson, 1995), and can also accelerate flowering in a monocot, rice (He et al., 2000). The *LFY* gene is activated by application of GA (Blázquez et al., 1997, 1998) and the *LFY* promoter of at least two dicots contains a potential MYB-binding motif that is required for normal *LFY* promoter activity (Blázquez and Weigel, 2000). In addition, consistent with the role for *GAMYB* in regulating *LFY*, *LtLFY* is induced after *LtGAMYB* during the floral transition at the shoot apex of *L. temulentum* (Gocal et al., 1999, 2001).

Here, we describe three *GAMYB-like* genes from Arabidopsis, *AtMYB33*, *AtMYB65*, and *AtMYB101*, the proteins of which are capable of transactivating an α-amylase promoter in barley aleurone cells. We show that *AtMYB33* and *AtMYB65* are co-expressed in many tissues, but *AtMYB101* expression is restricted to the subapical pith cells of both vegetative and flowering plants and to the hypocotyl hook. We have used measurements of GA levels, together with manipulation of GA levels, to investigate the role of GA in regulating expression of this group of genes during elongation growth and flowering. At the shoot apex, the timing and pattern of expression of *AtMYB33* precedes and overlaps with that of the *LFY* gene and, furthermore, AtMYB33 binds in vitro to a specific sequence in the *LFY* promoter. Such findings are compatible with a GA signaling role of *GAMYB-like* genes in flowering.

RESULTS

Three *GAMYB-like* Genes in Arabidopsis

Sequences of three Arabidopsis *GAMYB-like* genes (*AtMYB33*, *AtMYB65*, and *AtMYB101*) were obtained on the basis of sequence similarity to *GAMYB* genes from barley, rice, and *L. temulentum* (Gubler et al., 1995, 1997; Gocal et al., 1999). The nucleotide sequences of the three *AtMYB* cDNAs have been lodged with GenBank (accession nos. AF411969 for *AtMYB33*, AF048840 for *AtMYB65*, and AF411970 for *AtMYB101*). Based on cladistic analysis of partial sequences of more than 80 different AtMYB proteins, Kranz et al. (1998) defined a subgroup, number 18,
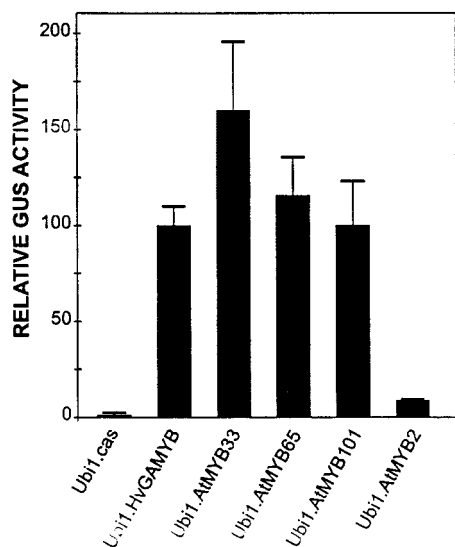
Figure 2. Transactivation of a barley α-amylase promoter by Arabidopsis GAMYB-like proteins. Intact aleurone cells were cobombarded with a reporter construct containing an α-amylase promoter fused to GUS and different Ubi1.MYB effector constructs: Ubi1.cas (a no-insert control), Ubi1.HvGAMYB, Ubi1.AtMYB33, Ubi1.AtMYB65, Ubi1.AtMYB101, and Ubi1.AtMYB2. The Ubi1.cas expression cassette is described by Gubler et al. (1999) The bars are twice the SE of the mean for the eight to 16 replicates per assay.

which comprised these three genes, *AtMYB33*, *AtMYB65*, and *AtMYB101*, and a fourth, *AtMYB81*, which we have not studied. The graminaceous *GAMYB*s are most closely related to the four members of this Arabidopsis subgroup. All share a QRaGLPxYPx(E/S) motif (Fig. 1, Box 1) immediately C-terminal to the R2 R3 repeat DNA-binding domain (Kranz et al., 1998; Romero et al., 1998). AtMYB33 has the highest overall identity with HvGAMYB (41%) but, over the R2R3 repeat sequence alone, identity with HvGAMYB was 86.4%, 85.4%, and 82.5% for AtMYB33, AtMYB65, and AtMYB101, respectively. In addition, there are regions around amino acids 371 to 387 and at the C terminus that are conserved between cereal GAMYBs and AtMYB33, AtMYB65, and AtMYB101 (Fig. 1, Boxes 2 and 3). Other regions are conserved between a subset of the Arabidopsis genes and cereal GAMYBs (Fig. 1). The genomic structure of Arabidopsis *GAMYB-like* genes is also conserved with *HvGAMYB* (F. Gubler and R. Kalla, unpublished data), with the location of an intron at the 3′ end of the open reading frame being unique to this class of *MYB* genes (Fig. 1). *HvGAMYB* has one intron in the 5′-untranslated region and *AtMYB33* has two (G.F.W. Gocal, F. Gubler, R. Kalla, and C.C. Sheldon, unpublished data).

AtMYB33 is located on the top of chromosome 5 at approximately 1,840,000 bp, *AtMYB65* on the top of chromosome 3 at approximately 3,610,000 bp, and *AtMYB101* at approximately 14,065,000 bp, two-thirds of the way along chromosome 2.

Functional Analysis of the Three *AtMYB* Genes

In transient expression experiments, *HvGAMYB* can substitute for GA$_3$ in activating transcription of the GA-responsive α-amylase promoters from barley (Gubler et al., 1995, 1999). We therefore determined whether this α-amylase promoter could be activated by expression of *AtMYB33*, *AtMYB65*, and *AtMYB101*. Barley aleurone tissue was bombarded with a low-pI α-amylase reporter along with each of the MYB effector plasmids. As a control for the specificity of GAMYB activity, we also assayed the ability of an unrelated Arabidopsis *MYB* gene, *AtMYB2* (Urao et al., 1998), to activate the α-amylase promoter. The α-amylase promoter was strongly induced by AtMYB33, AtMYB65, and AtMYB101, but not by AtMYB2 (Fig. 2). The increase in GUS activity in response to AtMYB33, AtMYB65, and AtMYB101 was similar to that observed with HvGAMYB.

Expression Patterns of Arabidopsis *GAMYB-like* Genes

We used RNase protection to analyze the tissue-specific expression pattern of the *AtMYB* genes in the Columbia ecotype (Fig. 3). The expression of *AtMYB65* was approximately 10-fold less than that of *AtMYB33* or *AtMYB101*. *AtMYB33* was expressed in all tissues, but it also showed a predominantly floral expression pattern. *AtMYB65* expression was fairly similar across all tissues although with a slight increase in floral tissues.

The expression patterns of the three genes were examined in more detail by in situ hybridization. In germinating seeds, *AtMYB33* RNA was found in the root tip and in a linear array of up to 20 to 30 cells above the root tip (Fig. 4 A). The vegetative shoot apex showed weak expression of *AtMYB33* both in germinating seeds and 60-d-old plants held in SDs (Fig. 4, C and E). Higher levels of expression were evident in primordial leaves which probably include developing petioles (Fig. 4E). As a control for speci-
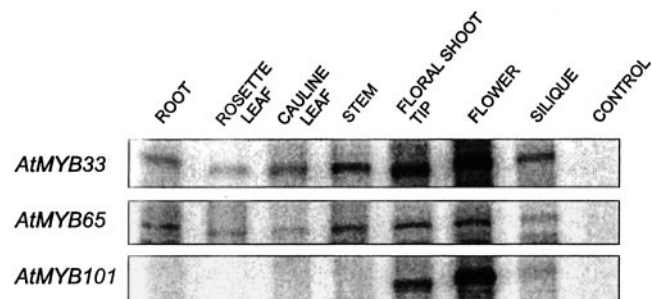

Figure 3. Expression pattern of Arabidopsis *GAMYB-like* genes, *AtMYB33*, *AtMYB65*, and *AtMYB101*, as determined by RNase protection assays of mRNA levels in various tissues from Columbia plants. The negative control for the RNase protection assay contained only yeast tRNA. The floral shoot tip included flower clusters at the tip of the inflorescence but with no visible petals.
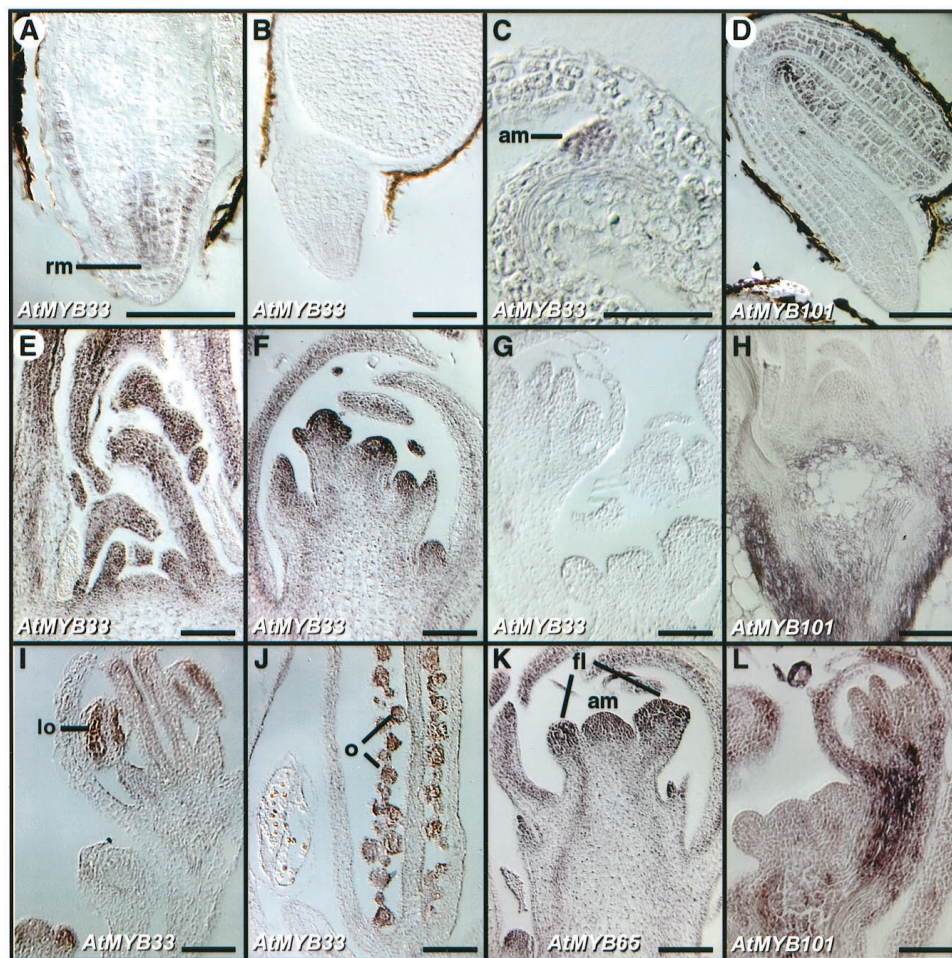
Figure 4. Localization by in situ hybridization of expression in Arabidopsis of *GAMYB-like* gene transcripts of *AtMYB33*, *AtMYB65*, and *AtMYB101*. Germinating seeds (A–D, Columbia wild type) were harvested 48 h after imbibition. Shoot samples were from vegetative (E and H) or florally induced (F, G, I, and L) plants. The shoot tissue was either from Columbia (H, J, and L) or Col *hy4-101* (E, F, G, I, and K), however, there were no differences between the genotypes in expression patterns in specific tissues (not shown). Control sections hybridized with *AtMYB33* sense probes are shown in B and G. Probe concentrations, DIG antibody concentration, and duration of color development were identical in all panels. Bars = 100 μm. am, Apical meristem; fl, flower primordia; lo, developing stamen locule; o, ovule; rm, root meristem.

ficity, there was no hybridization of *AtMYB33* sense probes as shown in Figure 4, B and G.

Inflorescence and flower primordia had developed to floral stages 2 to 3 (Smyth et al., 1990) at 6 d after transfer of plants to LDs. Compared with the vegetative shoot apex, *AtMYB33* was strongly expressed in the inflorescence apex (Fig. 4F). Its expression was comparatively weaker in the inflorescence stem, the vascular tissue, and the vascular tissue in leaf primordia. In the gynoecium only ovules showed expression (Fig. 4J), and in developing anthers *AtMYB33* was expressed in developing locules of immature anthers and later, albeit weakly, in pollen grains (Fig. 4, I and J). The expression pattern of *AtMYB65* in the root apex (not shown) and vascular strands and floral apices of the developing inflorescence (Fig. 4K) paralleled that of *AtMYB33*.

The pattern of *AtMYB101* expression was very different from that of *AtMYB33* and *AtMYB65* (Fig. 3).

There was little or no expression of *AtMYB101* in the root tip of germinating seedlings (Fig. 4D), in the vegetative (Fig. 4D), early floral (Fig. 4H), or inflorescence shoot apex (Fig. 4L), or in immature anther and carpel tissue (not shown). Expression of *AtMYB101* was detected in a small patch of cells on the innermost side of the hypocotyl hook of the germinating seedling (Fig. 4D), in the subapical pith cells of plants growing vegetatively (Fig. 4H), in a similar zone of expanding cells both in developing inflorescence stems (Fig. 4L), and below mature flowers and elongating siliques (not shown).

Effects of GAs on Petiole Elongation

In plants such as spinach, exposure to LDs enhances growth and erectness of petioles, and there is an accompanying increase in GAs (Zeevaart, 1971; Talón et al., 1991; Zeevaart and Gage, 1993). Here we
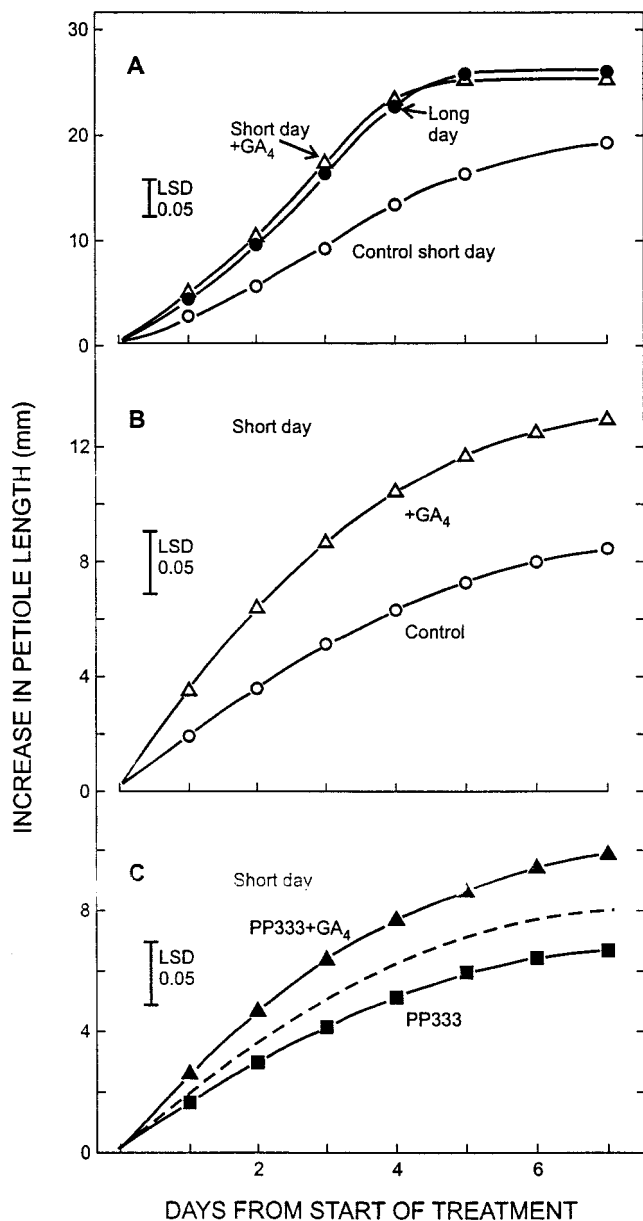
Figure 5. Arabidopsis petiole elongation in response to exposure to LDs or GA (millimeter increase over the starting length). A, control plants in SDs (about 60-d old plants of Col *hy4-101*; ○); transferred to LDs at the start of treatment (●); in SDs but treated once with GA_4 (15 μg in water applied to the shoot tip; △). B and C, Application in SDs of GA_4 (△; 66 ng in 95% ethanol applied to the measured petiole); Paclobutrazol (■; abbreviated as PP333), a GA biosynthesis inhibitor (200 ng in 95% ethanol), or 66 ng of GA_4 plus 200 ng of paclobutrazol (▲). Control plants were treated with water (A) or 95% ethanol (B and C). The dotted line in C is the relevant control from B. Bars are the value of the LSD at $P = 0.05$. There were eight replicate plants per measurement.

show comparable responses in Arabidopsis associated with LD exposure, which caused young petioles to grow faster and to become more erect. These responses occurred soon (within 1–2 d) after transfer of plants of Col *hy4-101* from SDs to LDs, their length at

6 d being about 1.5 times that of plants kept in SDs (Fig. 5A). The angle of the petiole to the horizontal almost doubled over the same 6-d period ($12.5 \pm 0.5°$ to $20.5 \pm 0.8°$).

In three ways these findings suggest that GA mediates the LD growth response. First, a single application of GA to SD-grown plants mimicked the effect of LDs on petiole elongation (Fig. 5). The elongation response differed between the SD controls in the two experiments shown (A versus B) but GA response equal to the LD response was found in both experiments (LD not shown for the experiment in Fig. 5B). Second, blocking GA synthesis with paclobutrazol inhibited the LD increase in petiole length, the increment at 3 d being 4.5 ± 0.7 mm in LD, 2.1 ± 0.2 mm with paclobutrazol application in LD, and 2.5 ± 0.4 mm in SD. Such effects of the chemical were specific because, in SDs, the 40% reduction in petiole elongation by paclobutrazol was reversed by application of GA_4 (Fig. 5C). Third, in the same experiments, LD-induced elongation of young petioles was matched by substantial increases in petiole GA content as shown in Figure 6 and Table I.

The content of bioactive GA_1 and GA_4 increased within 2 d of transfer to LDs, and by 6 d had increased 30-fold for GA_1 and 6-fold for GA_4 (Fig. 6; Table I). These findings with single assays were confirmed in a second, more restricted analysis in which, after two LDs, the increase in GA_1 was 2.8-fold (Table I). The levels of GA_4 were comparable between the two experiments, however, for plants in SDs more GA_1 was detected in the second than the first experiment (i.e. 0.045 and 0.052 ng g^{-1} dry weight versus 0.013 ng g^{-1} dry weight; compare with Table I).

There were no large changes in precursors of GA_1 such as GA_{53}, GA_{44}, GA_{19}, or GA_{20} although two immediate precursors of GA_4, GA_{24} and GA_9, increased somewhat (Table I). Part of the GA_1 increase could reflect a reduced catabolism to GA_8 (Table I). However, the drop in the level of GA_8 in LDs was not
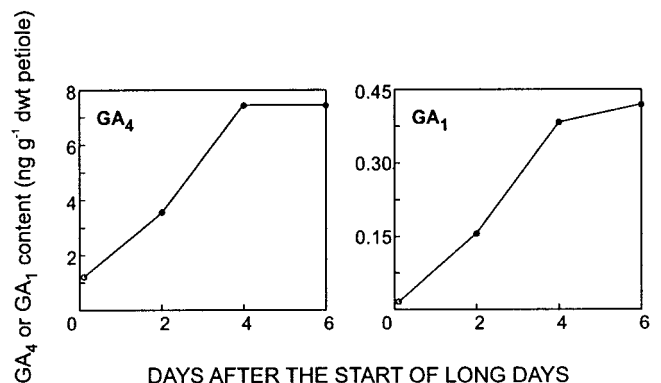

Figure 6. Increase in the endogenous GA content of young petioles after exposure of the plant to LDs. Young elongating petioles were harvested from SD plants (○) or at various times after exposure to LD (●) as part of the experiment detailed in Figures 4, 5A, and 7. Data for all 10 GAs assayed in this experiment is shown in Table I.

Table I. *GA content of young petioles of Arabidopsis*

Young petioles were harvested from 60-d-old plants grown in SDs or transferred at that time to LDs for 2, 4, or 6 d. Petiole elongation and flowering for experiment I are shown in Figure 5 and Table II.

Photoperiod Exposure	GA Content									
	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_1	GA_8	GA_{24}	GA_9	GA_4	GA_{34}
	ng g^{-1} dry wt									
Experiment I										
SD	24	4.4	18	0.8	0.01	8.4	52	2.0	1.2	3
2 LD	24	7.2	15	1.0	0.15	9.7	81	4.8	3.5	13
4 LD	27	9.0	16	1.3	0.37	3.4	103	4.1	7.3	22
6 LD	34	10.6	23	2.1	0.41	1.2	147	10.4	7.3	22
Experiment II										
SD	–	–	18	–	0.05	12	–	–	2.0	19
2 LD	–	–	19	–	0.14	13	–	–	2.7	45
4 LD	–	–	18	–	0.20	12	–	–	3.2	51

reproduced in the second experiment, a finding we cannot explain. Interestingly, compared with young petioles, no increase in GA levels could be detected in extracts of shoot tissue including leaves and old expanded petioles (data not shown), and this probably explains why Xu et al. (1997) could not detect any dramatic increase in LDs in GA_1 and GA_4 levels in their analysis of total shoot tissues of Arabidopsis.

Effects of LD and GAs on Flowering and *AtGAMYB* Expression

GA can enhance flowering of Arabidopsis and may be essential in SDs as demonstrated by Wilson et al. (1992) with a GA-deficient mutant. Furthermore, as shown in Table II, in the experimental conditions used here, flowering of Col *hy4-101* plants was induced equally well by a single application of GA_4 in SDs or by exposure to a single LD. However, such information on flowering is not definitive despite the parallel effects of GA on petiole elongation and on endogenous GA levels (Figs. 5 and 6). For flowering, we require information on shoot apex GA content but this is a challenging task because of the extremely small size of the shoot apex.

In the same set of plants used above for petiole growth and flowering, expression of the GA-responsive floral meristem-identity gene *LFY* was very low in the vegetative apex but increased dramatically after the 2nd d of exposure to LDs, i.e. during early inflorescence differentiation (Fig. 7, A–E). A comparable increase over time in expression of *AtMYB33* was evident during floral differentiation (Fig. 7, F–J). Apex height provided a more sensitive marker of floral response than the morphological floral stage shown in Figure 7. Apex height had increased after 2 d and probably before then (Fig. 7K). At these early times it was the apex alone that increased in height. Later (at 6 d) the new stem made up a larger component of the "apex" but all tissue was derived from the initially rather flat apex (Fig. 7, A, F, and K) rather than by activation of any preexisting subapical meristem, a finding quite unlike previous reports for other species (Sachs, 1965).

Application of GA_4 to plants in SDs induced flowering in our experiments (Table II), and *AtMYB33* expression increased with inflorescence differentiation (Fig. 8) in a parallel manner to that seen in Figure 7 for LD induction of flowering. Expression of *AtMYB33* in the immature anther locule (Fig. 4I) was evident also after GA application (C.P. MacMillan and R.W. King, data not shown).

One potential target in Arabidopsis for GAMYB action is the *LFY* promoter. Functional analysis of the *LFY* promoter has identified a cis-acting element critical for GA responsiveness (Blázquez and Weigel, 2000). Removal of this element within the context of a synthetic *LFY* promoter, referred to as GOF9, renders this *LFY* promoter insensitive to GA_3 and reduces its activity particularly in SDs. Within this cis-acting element there is a motif CAACTGTC (approximately −249 to approximately −242), which is a putative GAMYB-binding site based on the deduced consensus-binding site of GAMYB, which is $^C/_T$A-AC$^C/_G$$^G/_A$$^A/_C$$^C/_A$ (Gubler et al., 1999).

To examine the specificity of the *LFY* promoter GAMYB-binding motif, the AtMYB33 protein was expressed in *E. coli* and tested for its ability to bind to

Table II. *Induction of flowering of Arabidopsis, Col hy4-101, by LDs or GA treatment*

When retained for longer in SDs, these 60-d-old plants (*n* = 6) remained vegetative for many days but flowered when treated once with GA_4 (15 μg per plant) or when transferred for 1 or more d to LD conditions. Flowering time is shown as the number of days after d 60 that the first open flower was observed. Values are the mean ± SE.

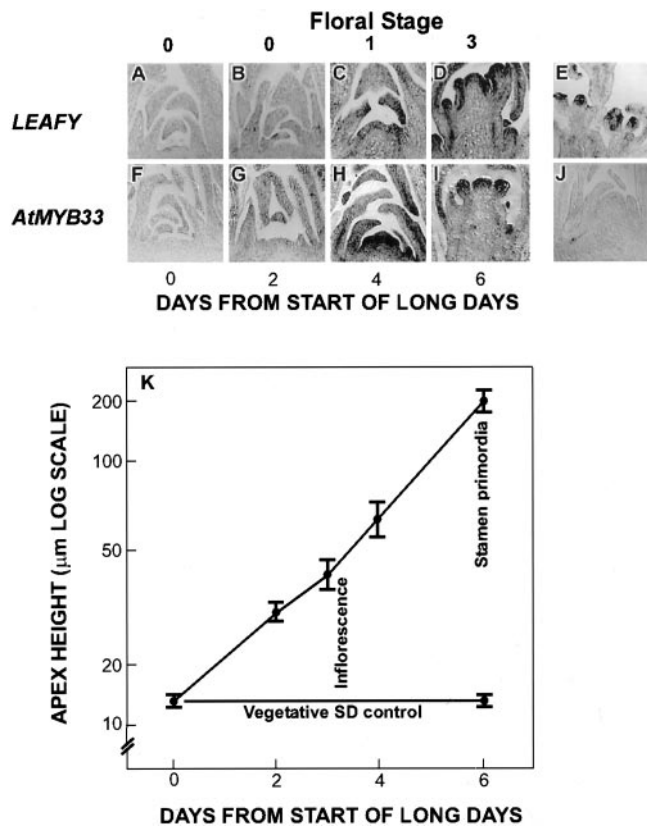| Treatment	Flowering Time
	d
SD	115 ± 8
SD + GA_4	41 ± 6
1 LD	42 ± 2
6 LD	17 ± 2

Figure 7. Increase at the shoot apex in expression of *AtMYB33* and *LFY* mRNA and change in shoot apex morphology over the early days of LD-induced flowering of Arabidopsis. Expression of *LFY* (A–E) and *AtMYB33* (F–J) was analyzed by in situ hybridization. Median longitudinal shoot apex sections are shown for: vegetative 60-d-old Col *hy4-101* plants growing in SDs (A and F); or during the floral transition after exposure to 24-h LDs for 2 d (B and G), 4 d (C and H), or 6 d (D and I). *LFY* expression at a later stage of floral development is shown in E. A sense control for *AtMYB33* is shown in J after 4 LDs. Floral scores are based on the scale proposed by Smyth et al. (1990). Change in shoot apex morphology during floral induction (shown in K) was determined by measuring shoot apex height (in micrometers) above a line joining the insertion point of the last two leaf primordia. The averages are based on the seven separate apices sectioned for *AtMYB33* mRNA localization. Bars indicate two times SE. The plants exposed to LD were part of the experiment shown in Figures 4 and 5.

a 287-bp fragment from the *LFY* promoter (position −375 to −88), which includes the putative MYB-binding site. AtMYB33 bound strongly to the 287 base promoter fragment causing a shift in electrophoretic mobility (Fig. 9). When six of the eight bases in the putative binding site were mutated, binding of AtMYB33 was reduced by more than 75% (Fig. 9).

Blázquez and Weigel (2000) reported that the same six base mutations in the context of the *LFY* GOF9 promoter caused a selective reduction of *LFY* promoter activity in SDs and removal of GA responsiveness. Taken together, these results suggest that AtMYB33 might influence flowering by mediating GA responsiveness of the *LFY* promoter.

DISCUSSION

A Group of Three *GAMYB* Genes in Arabidopsis

GAs regulate many developmental processes in plants, but only a few downstream effectors have been identified. One of these is the GAMYB transcription factor from barley. Here we have shown that the protein products of three *GAMYB*-related genes from Arabidopsis, *AtMYB33 AtMYB65*, and *AtMYB101*, are likely to transduce GA signals in this species.

Structurally, these three GAMYB-like proteins of Arabidopsis constitute a distinct subgroup that clusters most closely with *GAMYB*s from barley and other Gramineae and less so with other Arabidopsis MYBs containing an R2R3 DNA-binding domain (Gubler et al., 1995, 1997, 1999; Kranz et al., 1998; Gocal et al., 1999). Although the *GAMYB* clade in Arabidopsis has several members, to date, only one *GAMYB*-related gene has been found in each monocot species. Functionally, all the Arabidopsis genes readily substitute for the barley GAMYB in their transactivation of the barley α-amylase promoter (Fig. 2). By contrast, two structurally distinct MYB genes were inactive, *AtMYB2* (Fig. 2) and *C1* (Gubler et al., 1995).

AtMYB33 and *AtMYB65* may constitute a redundant gene pair with common functions in the plant as suggested by their similar expression patterns (Fig. 4). The divergent expression pattern of *AtMYB101* suggests it plays a different role(s). Because of expression of *AtMYB101* in subapical tissue but not in the shoot or root apex, we speculate that it may be involved in GA-regulated stem elongation. This would fit with the finding that GA application causes stem elongation (bolting) in association with enhanced cell division in subapical tissue in a number of rosette plants (Sachs, 1965) including Arabidopsis (Besnard-Wibaut, 1970).

GAs and Flowering

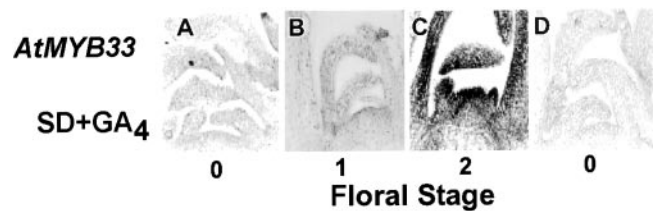GAs play an important role in mediating the flowering responses of several LD plants. One particu-

Figure 8. Increase in expression at the shoot apex of *AtMYB33* with GA$_4$ induced flowering of plants in SDs. Sixty-day-old plants were treated twice over 4 d with GA$_4$ (66 ng plant^{-1}). Expression of *AtMYB33* is shown at the start of treatment (A), at 8 d (B), or 12 d (C) and in D for a sense probe of an apex harvested 8 d after the start of treatment. Floral scores are based on the scale proposed by Smyth et al. (1990).
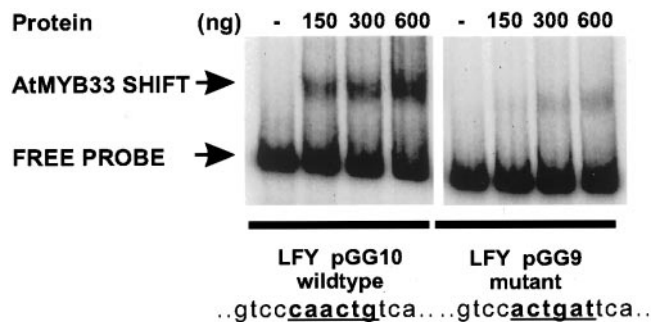
Figure 9. In vitro binding of AtMYB33 to the *LFY* promoter. At-MYB33 protein binding in vitro to a 287-bp ^{32}P-labeled fragment of the *LFY* promoter as seen by an electrophoretic mobility shift on a nondenaturing polyacrylamide gel. A 6-bp change (underlined) in the DNA sequence (pGG9) disrupts a putative GAMYB-binding site and reduces DNA binding by 75%.

larly well-studied example is with the LD and GA-responsive grass, *L. temulentum* (Evans et al., 1990; King et al., 1993). The content of GA_1 and GA_4 in *L. temulentum* leaves (Gocal et al., 1999) and the shoot apex (King et al., 2001) increases with exposure to two or more LDs, and there is a related up-regulated expression of *LtGAMYB* at the shoot apex (Gocal et al., 1999). Thus, these endogenous GAs may regulate the GAMYB expression seen at inflorescence formation. Similarly, GAs may be important for flowering of Arabidopsis because the GA-deficient mutant *ga1-3* only flowers in SDs if GA is applied (Wilson et al., 1992). In LDs, GAs are still important but to a lesser extent because there is still a significant delay in flowering of the *ga1-3* mutant (Wilson et al., 1992), and LD responsiveness of the *LFY* promoter is attenuated in this mutant (Blázquez et al., 1998). Here we have provided more certain evidence of a link between flowering, GA content, and changes in downstream molecular events.

Our claim of a link between GA and flowering is based principally on five observations: (a) A single GA_4 application induced flowering in SDs (Table II). (b) When LDs caused flowering (Fig. 7) there was a simultaneous increase in endogenous GA_1 and GA_4 in the petiole (Table I; Fig. 6) and, we assume, also in the shoot apex. (c) In association with flowering LD exposure led to enhanced expression at the shoot apex of *LFY* and *AtMYB33* (Fig. 7). (d) When GA_4 was applied, it induced expression of *AtMYB33* at the shoot apex in association with flowering (Fig. 8). (e) The promoter of the *LFY* gene contains an *AtMYB33*-binding motif (Fig. 9), which therefore provides a basis for linking GA to *LFY* expression.

There was an immediacy in these responses to LDs in that changes were evident over the first 2 d of floral induction. Simultaneously, there were increases in the size of the prefloral shoot apex (Fig. 7), in endogenous GA content of petioles (Fig. 6), and in petiole length and erectness (Fig. 5). Presumably, LDs caused GA content to increase in the shoot apex

as rapidly as in the young petiole, a presumption supported by the evidence of increased expression of *AtMYB33*, which is apparently a GA-regulated change (e.g. Fig. 8). We also believe that a LD-induced increase in GA content of the Arabidopsis shoot apex is likely given our recent finding of changes in GA content of the shoot apex of *L. temulentum* after exposure to LD(s) (King et al., 2001). Previous studies of effects of daylength on Arabidopsis have shown little or no change in GA content due to LDs, but this was for whole shoots (Xu et al., 1997), a tissue mix in which we also found no detectable change in GA content (R.W. King, unpublished data).

Our findings are problematic when considering the studies with a GA biosynthetic mutant by Wilson et al. (1992) that were recently confirmed by Reeves and Coupland (2001). On the one hand, these genetic studies imply regulation of flowering of Arabidopsis by a LD-independent, GA-dependent pathway and, separately, by a LD-dependent, GA-independent pathway (Piñeiro and Coupland, 1998). On the other hand, our studies here with Arabidopsis and earlier with the LD-responsive grass *L. temulentum* (King et al., 2001), strongly link flowering, LD exposure, and GAs. Possibly some of the reports of LD-dependent, GA-independent flowering (Blázquez et al., 1998; Piñeiro and Coupland, 1998, and refs. therein; Blázquez and Weigel, 2000) can be explained as photosynthetic effects of LDs, a suggestion supported by our recent evidence that photosynthetic Suc input limits flowering in *phyA* mutants of Arabidopsis (Bagnall and King, 2001). Nevertheless, where low-irradiance LD photoperiods are employed as were here or in the studies of Wilson et al. (1992) and Reeves and Coupland (2001), there is no likelihood of a LD increase in photosynthetic input. Perhaps, therefore, in LDs there are both GA-dependent (our study) and GA-independent (Wilson et al., 1992; Reeves and Coupland, 2001) responses, with photosynthetic input being a further component of the LD, GA-independent pathway(s).

As an aside, it is well known that GA regulates petiole elongation and plant habit in a number of plant species and, as shown here, also in Arabidopsis. Such change in morphology induced by GA has been particularly well documented for spinach (Zeevaart, 1971; Metzger and Zeevaart, 1985; Talón et al., 1991). There may be a GAMYB signaling role in such elongation growth in Arabidopsis, but we were unable to detect in petioles any change in expression of the three *AtMYB* genes during GA or LD-enhanced elongation (C.C. Sheldon, unpublished data). Similarly, although the differences in tissue specific expression patterns shown in Figure 4 might suggest roles for *AtMYB33* and *AtMYB65* in GA regulation of germination, testing this possibility has been difficult, particularly in the absence of mutants.

Another issue raised by our study relates to the nature of the first events of flowering. Despite the

evidence of a link between applied GA and expression of the floral regulatory gene, *LFY* (Blázquez et al., 1998; Blázquez and Weigel, 2000), and of *AtMYB33*, it must be questioned whether *LFY* or *AtMYB33* regulate any of the events during the first 2 LDs. In our studies, expression of these two genes had barely increased after 2 LDs when the apex was already progressing to flowering as shown by its increase in size (Fig. 7). Perhaps, for Arabidopsis, *LFY* and *GAMYB* expression is more associated with later inflorescence formation, as also reported in earlier studies with *L. temulentum* (Gocal et al., 1999, 2001). In previous work with Arabidopsis, *LFY* was expressed early during floral development and even in the shoot apex of vegetative plants (Blázquez et al., 1997; Hempel et al., 1997). However, compared with the domed apex of the "vegetative" plants used in earlier studies (e.g. Blázquez et al., 1997; Hempel et al., 1997), our vegetative plants had a very flat apex that was essentially devoid of *LFY* expression (e.g. Figs. 4, 7, and 8). Thus, it is possible that in these earlier studies, the shoot apex had already progressed developmentally beyond the earliest events of "floral evocation."

Expression of *LFY* during the inflorescence transition implies late GA-regulation but does not exclude a separate, earlier action of GAs on flowering. In this context, our recent measurements of the content of GAs at the shoot apex of *L. temulentum* (King et al., 2001) highlight two such GA actions during flowering. Following the 1st d of LD exposure (i.e. at floral evocation of *L. temulentum*), the GA_5 content of the apex doubled, after which expression increased for two floral-specific *APETALA1*-related genes (Gocal et al., 2001). A few days later at inflorescence initiation, there were large increases in the content of bioactive GA_1 and GA_4 (King et al., 2001), after which there was increased expression of *LtGAMYB* (Gocal et al., 1999) and of *LtLFY* (Gocal et al., 2001). Thus, as for *L. temulentum*, our evidence with Arabidopsis is consistent with applied or endogenous GAs acting as regulators of *LFY* expression and, hence, of flowering, but increased LFY expression is not necessarily the earliest "floral" response to GAs.

GAs, Flowering, and a GAMYB/LEAFY Signaling Pathway in Arabidopsis

The scenario of GA regulating flowering via its activation of *LFY* gene expression raises the possibility that transcriptional regulators involved in the GA signal transduction pathway might be regulators of *LFY*. *AtMYB33* is a candidate for such a regulator. Not only could it replace GA in activating the α-amylase promoter in transient expression assays (Fig. 2), but also its expression increased at the shoot apex (Fig. 7, 8) in association with GA application (Fig. 8) or with increased endogenous GA levels in the plant (Fig. 6). However, most cogently, we have

identified a potential regulatory hierarchy involving AtMYB33 binding to a GA-responsive region of the promoter of the floral meristem-identity gene *LFY* (Fig. 9). Recent studies of Blázquez and Weigel (2000) provide further support for such a signaling hierarchy and for in planta relevance of our studies. They found that the same mutation in the *LFY* promoter that we used to block AtMYB33 binding also blocks GA-induced expression of a LFY-GUS reporter gene construct.

Overall, our studies extend our earlier evidence (Gocal et al., 1999) that the action of GA on flowering may be via the *GAMYB* class of transcriptional regulators and, potentially, via transactivation of a specific floral regulatory gene, *LFY*.

MATERIALS AND METHODS

Plant Material, Growing Conditions, and Chemical Treatments

Plants of Arabidopsis ecotype Columbia wild-type or a blue-photoreceptor (cryptochrome 1) mutant (Col *hy4-101*) were grown in soil in an 8-h short day (SD) at an irradiance of 100 μmol photons m^{-2} s^{-1} at 22°C. In these conditions involving exposure to blue-rich fluorescent tubes, *hy4-101* plants took up to 26 weeks to flower. However, for flowering its phytochrome responses are unaltered compared with Columbia (Bagnall et al., 1996). Thus, in the experiments here (see below), we could induce flowering within 1 to 2 d on transferring 8-week-old plants to 24-h LDs given as a 16-h extension at low irradiance (10 μmol photons m^{-2} s^{-1}) from far-red-rich incandescent lamps. Such a combination of genetics and photophysiology with its focus on LD phytochrome inputs also avoided the often substantial LD photosynthetic effects on flowering that we have recently documented in Arabidopsis (Bagnall and King, 2001).

GAs were synthesized and supplied by Prof. Lewis. N. Mander (Research School of Chemistry, Australian National University, Canberra, Australia). The GA biosynthesis inhibitor, paclobutrazol ([2*S*,3*S*]-1-[4-chlorophenyl]-4,4-dimethyl-2-[1,2,4-triazol-l-yl] pentan-3-ol), was obtained as the pure enantiomer from Dr. J. Lenton (Long Ashton Research Station, UK). Chemicals were applied directly to shoot tips in 40 μL of aqueous solution and included 5% ethanol and 0.1% Tween 20 as a wetting agent. Alternatively, chemicals were applied directly to young petioles as a 2-μL drop in 95% ethanol. Controls were treated with the same solution without the test chemical.

Isolation of cDNA and Genomic Clones

Cloning of AtMYB33

A reverse transcription-PCR (RT-PCR) was carried out to generate a cDNA library using RNA from Arabidopsis ecotype Columbia hy4-101 shoot tips as starting material. The AtMYB33 cDNA clone was identified using an LtGA-MYB fragment (nucleotides 1–567 of the coding sequence;

Gocal et al., 1999) as probe at intermediate stringency (0.5×
SSPE [sodium chloride/sodium phosphate/EDTA]; 0.2%
SDS at 65°C). Full-length cDNA and genomic clones were
isolated from a Landsberg *erecta* (L*er*) floral cDNA library
(Weigel et al., 1992) and from a L*er* genomic library using
a 3′ gene-specific AtMYB33 fragment (nucleotides 1023–
1848 of the cDNA). The filters were washed at high strin-
gency (0.1× SSPE, 0.2% SDS at 65°C).

Cloning of AtMYB65

A genomic DNA library from L*er* was probed with the
degenerate 38-mer oligonucleotide 5′-CCTGGTCGTACT-
GA(C/T) AA(C/T) GA(A/G) ATTAA(A/G) AA(C/T)
TA(C/T) TGGAA-3′, which corresponds to the conserved
region of the MYB R3 repeat. A cDNA clone was isolated
by RT-PCR using floral poly(A⁺) RNA.

Cloning of AtMYB101

A BLAST search of GenBank with the barley (*Hordeum
vulgare*) *GAMYB* sequence identified the genomic sequence
ATM1 (accession no. X90379; Quaedvlieg et al., 1996),
which contains the promoter region and the R2R3 domain
of a MYB gene. An *AtMYB101* cDNA clone and a genomic
clone were isolated from a L*er* floral library (Weigel et al.,
1992) and a C24 ecotype genomic library, respectively,
using a 271-bp PCR product covering the R2R3 sequence of
ATM1. Genetics Computer Group software (version 10,
Madison, WI) was used for sequence analysis.

RNase Protection

Total RNA was extracted from approximately 0.5 g of
plant tissue, following the method of Logemann et al.
(1987). Templates for RNA probe preparation were gener-
ated by PCR amplification of the 3′ region of each gene,
containing part of the 3′ intron and the 3′ coding region.
The primers were: AtMYB33F(*Bam*HI), 5′-CGCGGATCCA-
CACAAAATGCAGATG-3′; AtMYB33R(*Kpn*I), 5′-CGG**GG-
T**ACCAATGGAGTGGAGGAT-3′; AtMYB65F(*Bam*HI), 5′-
CGC**GGATCC**CTCGCAACTTAGTGC-3′; AtMYB65R-
(*Kpn*I), 5′-ACGGGGTACCGTTACAGCGACCAAACAG-3′;
AtMYB101F(*Bam*HI), 5′-CGC**GGATCC**TTCTCATCAT-
TCATCATTG-3′; and AtMYB101R(*Sal*I), 5′-ACGC**GTC-
GAC**GTTCCAAACTAACAGATGC-3′.

The PCR products were digested with the restriction
enzymes indicated in the primer name (restriction sites
indicated in bold, annealing sequence underlined) and
cloned into the appropriately digested pBluescript SK and
KS (Stratagene, La Jolla, CA). Constructs were sequenced
to ensure their identity. RNA probes were synthesized
using a *Bam*HI-linearized pBluescript SK-AtMYB33, 65 and
101 plasmid, for antisense transcript probe or a *Kpn*I-
linearized pBluescript KS-ATMYB33 for a sense transcript
probe and T7 RNA polymerase (Promega, Madison, WI)
according to the manufacturer's protocol.

RNase protection assays were carried out using the Hyb-
speed RPA kit (Ambion, Austin, TX) with 10⁵ cpm of the

appropriate riboprobe and 20 µg of total plant RNA fol-
lowing the manufacturer's protocol. Protected fragments
were precipitated, separated on 5% polyacrylamide/8 M
urea gels and analyzed with a PhosphorImager (Molecular
Dynamics, Sunnyvale, CA). The assays were run three
times and gave similar results each time.

In Situ RNA Localization

Seeds of Columbia were imbibed on water-soaked filter
paper in the dark at 4°C for 2 d and then germinated at
22°C under continuous light. After 48 h the radicle first
protruded, and seeds were then fixed for sectioning (Fer-
rándiz et al., 2000). For vegetative and floral shoot apices,
60-d-old plants of Col *hy4-101* were induced to flower by
transfer from SD for between 1 and up to 6 LDs. On
longitudinal sections of the shoot apex, the first signs of
transition to flowering were evident, microscopically, after
as few as 2 LDs. Maturing pollen-stage flowers were ob-
tained from Columbia wild-type and *hy4-101* plants. In situ
hybridization was as described by Ferrándiz et al. (2000).
For the Arabidopsis *LFY* gene the probe pDW119 was used
(Weigel et al., 1992). For the probes for each of the three
AtMYB genes a 3′ non-cross-hybridizing region of each
gene was generated as detailed below.

An AtMYB33-specific *Eco*RI/*Sma*I fragment was sub-
cloned into the *Eco*RI/*Eco*RV sites of pBluescript SK⁺ and
KS⁺. A 450-bp region of the 3′ AtMYB65 cDNA sequence
was amplified by PCR with primers 5′-GG**ACTAGT**GCA-
GGGAATGTTGTAAAG-3′ and 5′-CCG**CTCGAG**TATAT-
ATAAATGCCTTCA-3′ (restriction enzyme sites in bold,
annealing sequences underlined) using the expressed se-
quence tag 166K23 as template. The PCR product was
digested with *Xho*I and *Spe*I and cloned into *Xho*I and *Spe*I
digested pBluescript SK and KS.

A 330-bp region of the 3′ AtMYB101 cDNA sequence
was amplified by PCR with the primers; 5′-*AGTGGA-
ATTC*TTATGGGAAACC-3′ and 5′ACGG**GGTAC**CTCATT-
CCTCATCTCTTTCA-3′ (restriction enzyme sites in bold,
annealing sequences underlined) using the AtMYB101
cDNA clone as template. The PCR product was digested
with *Eco*RI and *Kpn*I and cloned into *Eco*RI- and *Kpn*I-
digested pBluescript SK and KS. Sense and antisense DIG-
labeled in vitro-transcribed riboprobes were synthesized
from the T7 promoter of AtMYB33 subclones linearized
with *Eco*RI, AtMYB65 subclones linearized with *Xho*I and
*Spe*I, respectively, or AtMYB101 3′ subclones linearized
with *Kpn*I and *Eco*RI, respectively

DNA Constructs for Transient Expression Assays

The maize (*Zea mays*) ubiquitin gene expression cassette,
Ubi1.cas, and Ubi1.HvGAMYB have been previously de-
scribed (Gubler et al., 1999). PCR-generated fragments
were inserted into Ubi1.cas to create Ubi1.AtMYB33 and
Ubi1.AtMYB101. The *Hin*cII/*Sma*I fragment of the full-
length AtMYB65 cDNA was inserted into the *Sma*I site of
pUbi1.cas resulting in Ubi1.AtMYB65. An AtMYB2 cDNA
(a gift from R. Dolferus, Commonwealth Scientific and

Industrial Research Organization, Canberra, Australia) was cloned into Ubi1.cas to generate Ubi1.AtMYB2. The mlo22 construct containing the barley low-pI α-amylase promoter β-glucuronidase (GUS) reporter has been described (Lanahan et al., 1992).

Transient Expression Analyses

For cobombardment experiments, 1 μg of effector and 2 μg of reporter plasmid were precipitated onto 3 mg of gold as described (Gubler et al., 1999). Following bombardment of barley half-grains (*Hordeum vulgare* L. cv Himalaya), they were incubated in 2 mL of medium without hormone (10 mM CaCl$_2$, 150 μg mL^{-1} cefotaxime, and 50 IU mL^{-1} nystatin). After 24 h at 25°C, the bombarded half grains were frozen and stored at −20°C. Extract preparation and GUS activity assays have been described (Lanahan et al., 1992).

In Vitro DNA Binding

AtMYB33 protein with an amino-terminal GST and carboxy-terminal hexa-His was expressed in *Escherichia coli* BL21-DE3 cells and affinity-purified over a nickel-nitrilotriacetic acid agarose column (Qiagen, Valencia, CA). This expression construct (pGG19) was a derivative of pGEX4T3 (Pharmacia, Piscataway, NJ). The region between −375 and −88 of the *LFY* promoter was subcloned between the *Pst*I and *Xho*I sites of pBluescript KS$^+$ to create pGG10. A mutated construct, pGG9, contains the same region as in pGG10, but has the MYB-binding site mutation (GTCCatcgatTCAATTT) found in GOF9 m (Blázquez and Weigel, 2000). Inserts between *Not*I and *Xho*I from pGG9 and pGG10 were gel purified and end filled with [^{32}P]dCTP using Klenow. In vitro DNA binding was carried out as described by Parcy et al. (1998).

Analysis of Endogenous GA Content

Methods for GA extraction were as outlined by Gocal et al. (1999). The smallest samples, young petioles, had dry weights of 0.1 to 0.5 g. Dideuterated internal standards were supplied by Prof. L. N. Mander (Research School of Chemistry, Australian National University, Canberra, Australia). After HPLC and methylation, the purified samples were passed through an amino column, silylated, and analyzed by high-resolution gas chromatography-mass spectrometry with selected ion monitoring (Moritz and Jensen, 1995; King et al., 2001).

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