Floral determination and expression of floral regulatory genes in *Arabidopsis*

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SUMMARY

The expression of the floral regulators *LEAFY*, *APETALA1*
and *AGAMOUS-LIKE8* was examined during light treat-
ments that induced flowering in *Arabidopsis,* and was
compared to time points at which floral determination
occurred. Extension of an 8-hour day by either continuous
red- or far-red-enriched light induced *LEAFY* and
AGAMOUS-LIKE8 expression within 4 hours. The 4 hours
of additional light was sufficient for floral determination
only in the far-red-enriched conditions, while 12-16 hours
of additional light was required for floral determination in
the red-enriched conditions. These results indicate that the
induction of floral regulatory genes and induction of flower

formation can be uncoupled under certain circumstances.
Expression of *LEAFY* and *AGAMOUS-LIKE8* in the shoot
apex at the time of floral determination is also consistent
with genetic data indicating that these genes are involved
in the first steps of the transition from vegetative to repro-
ductive development. In contrast to *LEAFY* and
AGAMOUS-LIKE8, *APETALA1* expression was first
observed 16 hours after the start of photoinduction. Since
this time point was always after floral determination,
APETALA1 is an indicator of floral determination.

Key words: *AGL8*, *APETALA1*, *LEAFY*, meristem, floral induction

INTRODUCTION

Alternating periods of light and dark, i.e., photoperiods,
modulate the flowering response of a wide variety of plants
(Garner and Allard, 1920; Thomas and Vince-Prue, 1997). In
many species, relatively brief photoperiodic changes can
trigger rapid flowering, provided that the plants are old enough
to respond to the treatment. For example, photoperiodic treat-
ments can induce competent *Arabidopsis thaliana, Ipomoea
nil*, *Lolium temulentum* and *Sinapis alba* plants to flower in less
than one day (King and Evans, 1969; Bernier et al., 1981;
McDaniel et al., 1991; Corbesier et al., 1996). Morphological
analysis of the transition to flowering in *Arabidopsis*, a facul-
tative long-day plant (Napp-Zinn, 1985), has shown that the
first primordium that is initiated from the shoot apex after the
start of inductive light treatments can adopt a floral fate
(Hempel and Feldman, 1994). When left in non-inductive con-
ditions, the same primordium will develop into a leaf with an
associated shoot (paraclade) instead.

The formation of normal flowers requires the expression of
flower-meristem-identity genes such as *LEAFY (LFY)* and
APETALA1 (AP1), which act as genetic switches in the choice
of floral versus shoot fate (Yanofsky, 1995). In *lfy ap1* double
mutants, most flowers are replaced by shoots with subtending
leaves, while overexpression of either *LFY* or *AP1* causes
lateral shoots to be converted into flowers (Weigel et al., 1992;
Mandel and Yanofsky, 1995b; Weigel and Nilsson, 1995). In
wild-type plants, *LFY* and *AP1* are expressed at high levels

early in the ontogeny of flower primordia, indicating a direct
role for these genes in specifying floral fate (Mandel et al.,
1992; Weigel et al., 1992). Since *LFY* and *AP1* are expressed
early during the development of individual flowers and shortly
after flowering is induced (Simon et al., 1996), they should also
be useful as molecular markers during the induction of
flowering.

Another potentially useful marker for early events in the
induction of flowering is *AGAMOUS-LIKE8 (AGL8)*, whose
sequence is very similar to that of *AP1* and the related *CAU-
LIFLOWER (CAL)* gene (Purugganan et al., 1995). Recent
genetic experiments indicate that *AGL8* acts redundantly with
AP1 and *CAL* to promote flower formation (C. Ferrandiz and
M. Yanofsky, unpublished results), and expression analyses
indicate *AGL8* is expressed in inflorescence meristems, inflo-
rescence stems, and cauline leaves, as well as in floral anlagen
(Mandel and Yanofsky, 1995a).

The goal of this study was to establish when floral determi-
nation in *Arabidopsis* occurs relative to changes in *AGL8*, *LFY*
and *AP1* expression. To this end, we used determination assays
in which plants were transiently exposed to photoinductive
conditions and then returned to non-inductive conditions
(Battey and Lyndon, 1990; Huala and Sussex, 1993). In
addition to analyzing the length of light treatment needed to
induce floral determination, we also studied the effects of two
very different ratios of red to far-red light. Since the red-to-far-
red ratio is known to affect the efficacy of floral induction
(Martínez-Zapater and Somerville, 1990; Goto et al., 1991;

Bagnall, 1993; Lee and Amasino, 1995), this provided us with a means to assess the generality of our observations regarding the relationships between floral determination and floral regulatory gene expression.

We found that only *AP1* expression was a qualitative marker for floral determination, while expression of *AGL8* and *LFY* was detected before floral determination. However, the expression patterns of *AGL8* and *LFY* also change, quantitatively, during the photoinduced transition to flowering. This work complements results presented in an accompanying paper (Blázquez et al., 1997), which documents gradual changes in *LFY* expression during the vegetative phase of plant development. In addition, we found that red-enriched (R) and far-red-enriched (FR) light conditions differed more in their ability to induce floral determination than in their ability to induce the early expression of floral regulatory genes.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds were sown in 3-inch pots on the surface of a 1:1 mix of Sunshine-mix (Fisons Horticulture Inc., Bellevue, Washington, USA) and vermiculite. Plants were grown at 22°C in Conviron growth chambers (Controlled Environments Ltd., Winnipeg, Manitoba, Canada). Each day the soil was soaked from below, and subsequently drained. The watering solution contained 0.12 g/l of an N:P:K (19:24:18) fertilizer with micronutrients (RA-PID-GRO; Chevron Chemical Co., San Francisco, CA, USA).

In the primary comparative experiment (Figs 1-5, 6A-D), plants were initially grown in short-days (8 hours light/16 hours dark) as a single population in two equivalent chambers. The plants were thinned to a density of six to eight plants per pot to prevent overlap of leaf rosettes and to enhance population uniformity. Pots were rotated within and between the two chambers to minimize the effects of minor light variation in the chambers. Irradiance during the pre-induction short-day treatment was 120-130 μmol/m^{2}second, with both chambers set for red-light-enriched (R) conditions (Table 1). Continuous-light photoinduction treatments started at the end of an 8-hour short-day, 25 days after germination; i.e., hour 0 of the photoinduction treatments occurred at 8 hours into a continuous light period. At the start of photoinduction, one of the chambers was set for far-red-enriched (FR) conditions (Table 1). Plants used in the determination assays were moved to a ventilated, light-tight room for their appropriate dark periods (see Fig. 1).

The following fluorescent bulbs were used in the primary experiment (see Table 1 for fluorescent bulb configurations and red-to-far-red ratios): Cool-White bulbs, which emit a wide spectrum of wavelengths, but emit minimally in the red and far-red regions of the light spectrum (Philips F48T12/CW/VHO; Philips Lighting Co., Somerset, NJ, USA); Gro-Lux Narrow Spectrum bulbs, which emit strongly in the red region and minimally in the far-red region (Sylvania F48T12/GL/NS/VHO; Osram Sylvania Inc., Danvers, MA, USA); 232 bulbs, which emit primarily in the far-red region of the spectrum (Sylvania F48T12/232/VHO).

In a pair of secondary photoinduction experiments (Figs 6E,F and 7), combinations of Cool-White and Gro-Lux Wide Spectrum bulbs were used. The red-to-far-red ratio in the latter experiments was approximately 2:1, and irradiance levels were 120-140 μmol/m^{2}second. For the in situ hybridization experiments, Landsberg *erecta* plants were stratified for 3 days at 4°C before being transferred to short-day conditions (8 hours light and 16 hours dark). Plants were kept under short-day conditions for 28 days and then transferred to continuous light at the end of the 8-hour light period on the twenty-eighth day (t = 0). The transgenic plants assayed in the second set of

experiments were stratified for 5 days at 4°C, and transferred to continuous light after 25 short-days.

Scanning electron microscopy and morphological analyses

Methods for scanning electron microscopy were as described by Hempel and Feldman (1994, 1995). The initiation of a primordium is defined as the point at which it becomes evident as a bump on the surface of the apical meristem (Esau, 1977). Flower and leaf primordia were distinguished as previously described (Hempel and Feldman, 1995). Chimeric flower/paraclade primordia (see Hempel and Feldman, 1995 for definition) were scored as paraclade primordia, although chimeric primordia and flower primordia could not always be differentiated at the 48 and 72 hour time points. Approximately half of the plants in both continuous-light control populations had a single chimeric shoot on the primary axis.

Determination assays

To define the length of photoinductive light extension required for floral determination, we used the following assay (see Fig. 1). After exposure to 4, 8, 12 or 16 hours of additional R- or FR-enriched light at the end of an 8-hour short-day, treatment populations of 25-day-old plants were returned to short-day cycles for 10 additional days. A pair of control populations was exposed to continuous light starting day 25 (R- and FR- continuous-light controls). A third control population (short-day control) remained in short days until it was placed in continuous FR conditions along with the treatment populations on day 35. To assess the effect of the different light extensions, we counted the number of leaves on the primary axis, a standard indicator of flowering time (Koornneef et al., 1991). Primary axis leaves were counted after bolting. Flower/paraclade chimeras were scored as having a subtending leaf, even if that leaf was not visible macroscopically (after Hempel and Feldman, 1995).

Photoinductive treatments that were sufficient for floral determination were those in which plants produced approximately the same number of leaves on their primary axis as the continuous-light controls. This indicated that the light-extension treatment induced the plants to cease leaf production and begin flower production. Conversely, plants that produced the same number of leaves on the primary axis as the short-day control were judged to have received treatments insufficient for floral determination (Fig. 1), as they did not produce flowers until they were photoinduced on day 35 with the short-day control.

To allow for the analysis of *LFY*, *AGL8* and *AP1* expression at all of the many timepoints included in the determination experiment (including many that are not specifically discussed), transgenic plants and GUS assays were utilized, instead of in situ hybridization of RNA.

Transgenic plants

The *AP1::GUS* fusion contains a restriction fragment that extends from a *Sac*I site approximately 1.7 kb upstream of the transcription start to a *Sac*I site within the untranslated leader region of the *AP1* gene (Mandel et al., 1992). After addition of *Bam*HI linkers, the fragment was inserted into the *GUS* fusion vector pBI101.2 (Clontech) (Jefferson et al., 1987). The *AGL8::GUS* fusion contains a 2.3 kb fragment of the *AGL8* promoter inserted into the *Hin*dIII/*Sal*I sites of pBI101.2. Each construct was introduced into Nossen plants by root transformation (Valvekens et al., 1988). The generation of *LFY::GUS* lines (containing pDW150) is described by Blázquez et al. (1997).

For each construct, a minimum of five independent lines was backcrossed three times into the Nossen background. Each line was assayed for GUS activity before and after the induction of flowering in preliminary experiments. Three representative lines were chosen for the photoinduction experiments (AM416.21 = *AGL8::GUS*, AM154.5c = *AP1::GUS*, DW150.12 = *LFY::GUS*). In preliminary experiments, all three lines were shown to initiate flowers equivalently in response to

Fig. 1. Experimental design of the determination assays. To assay for determination during the continuous-light induction of flowering, subpopulations of photoinduced plants were moved out of continuous-light conditions (R or FR) and returned to short-day conditions at 4-hour intervals. For simplicity, only two photoinduction treatments (4 hours of light extension and 8 hours of light extension) are shown. All populations, except the continuous light controls, were exposed to 10 additional short-days and then induced to flower in continuous FR conditions.
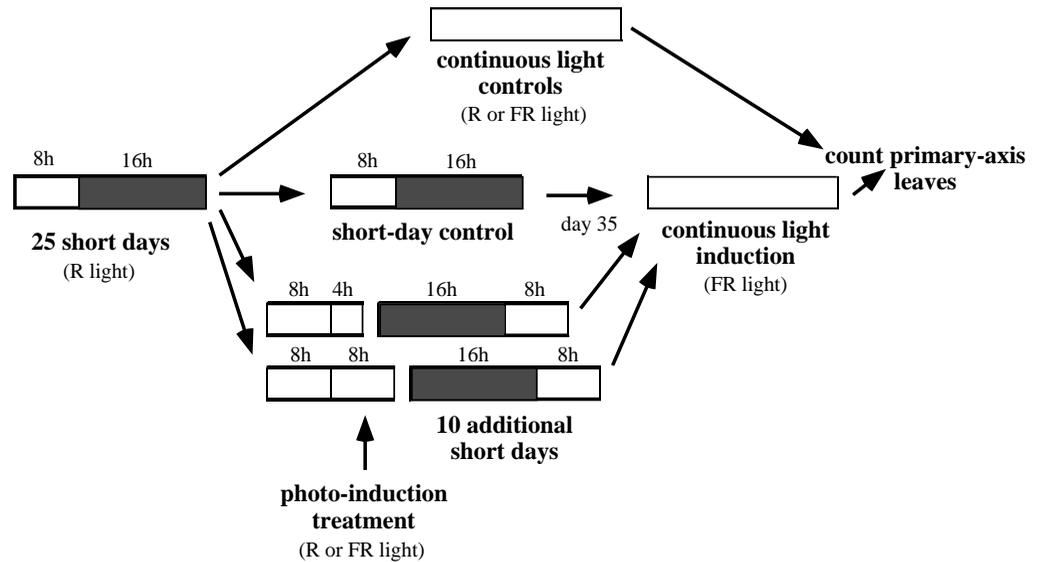
induction treatments, and to behave identically in floral determination assays. *LFY::GUS* apices are shown in Fig. 3 and the *LFY::GUS* line was used for the determination assays reported in Fig. 4.

GUS assays

For each plant, roots and larger leaves were removed. The remaining tissue was longitudinally bisected and placed in a 50 mM X-gluc solution (50 mM sodium phosphate buffer pH 7, 0.2% Triton X-100, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 20% methanol) on ice. The tissue was infiltrated in the dark under vacuum for 1 hour on ice and 2 hours at room temperature. The tissue was incubated for 20 hours at 30°C, in the dark; post-fixed (at 4°C) for 24 hours in a formaldehyde (3.7%)-alcohol (50%)-acetic acid (5%) fixative; dehydrated in a graded ethanol series (at 4°C); and embedded in paraffin. At least six apices were sectioned for each time point. All sections were cut at 15 µm, mounted and stored in the dark. Representative apices were photographed using a Leica DMRB microscope.

In situ hybridization

Tissue preparation and hybridization conditions were as described by Drews et al. (1991) with minor modifications. The generation of antisense RNA probes, which were used at a final concentration of approximately 2.5×10^7 cpm/ml, have been described previously (Mandel et al., 1992; Mandel and Yanofsky, 1995a; Blázquez et al., 1997).

RESULTS

Experimental design

The goal of this study was to relate the expression patterns of floral regulatory genes during the photoinduction of flowering to an assay-based developmental time point – floral determination. To eliminate variations between individual induction-of-flowering experiments, we assayed for reporter gene expression and floral determination within the same experiment. Consequently, it was possible to directly relate specific gene expression patterns to a given developmental state.

In the primary experiment, a single population of plants was germinated under uniform conditions in short days. After 25 days of vegetative growth, plants were divided into sub-populations and used either for morphological analyses, determination assays, or reporter-gene expression analyses. Transgenic lines containing *LFY::GUS*, *AGL8::GUS* and *AP1::GUS* reporter constructs (in the Nossen ecotype) were used for all three types of analysis. Experimental populations were induced to flower in two growth chambers that provided very different light quality, but that were otherwise identical (Table 1). One chamber had red-enriched (R) conditions (red-to-far-red ratio = 23.3:1), and the other had far-red-enriched (FR) FR conditions (red-to-far-red ratio = 0.6:1).

Effects of light quality on flower initiation and floral determination

Extension of an 8-hour short day with continuous light that was either R- or FR-enriched promoted the rapid initiation of flower primordia (Fig. 2). The number of initiated flower primordia per plant in the FR treatment was consistently higher (Figs 2, 3), indicating that flower primordia were produced more quickly in FR conditions. We could not pinpoint the precise

Table 1. Light conditions in the primary experiment

Light conditions	Fluorescent bulbs	Total irradiance	Red irradiance (680 nm)	Far-red irradiance (730 nm)	Red:far-red ratio
R	3 Cool-white 3 GroLux	120-130	1.4	0.06	23.3:1
FR	3 Cool-white 3 "232"	80-90	0.27	0.45	0.6:1

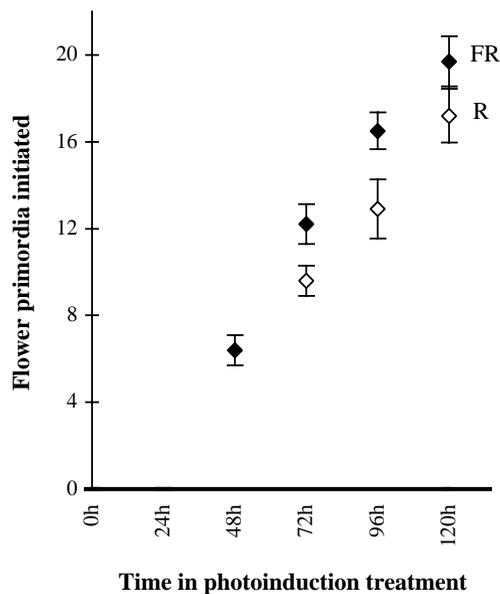Irradiance units = µmol/m²second.

Fig. 2. Initiation of flower primordia in the continuous-light control populations. Photoinduction treatments began 8 hours into a continuous-light period. Plants ($n = 10$/time point) were induced after exposure to 25 non-inductive short days (R light). Induced plants were exposed to R light for the first 8 hours of the continuous light photoperiod, and were subsequently exposed to either continuous R or FR light. Black diamonds = primordia initiated in continuous FR light; white diamonds = primordia initiated in continuous R light. Flower and leaf primordia were first distinguishable 48 hours into the FR treatment and 72 hours into the R treatment.

Fig. 3. Photoinduced morphogenesis at the *Arabidopsis* shoot apex. (A) Vegetative apex of a 25-day old plant. The apical meristem (asterisk) is surrounded by multiple leaf/paraclade primordia. The two youngest leaf/paraclade primordia are denoted by arrowheads. Note that in *Arabidopsis*, leaves and lateral flowering shoots (paraclades) differentiate from the same primordium – the leaf/paraclade primordium (Hempel and Feldman, 1995). (B) Shoot apex of a plant induced for 48 hours in FR light. The last leaf primordium initiated (arrowhead) is evident, as are six flower primordia (f1-f6) labeled in the order of their initiation from the meristem (asterisk). (C,D) Shoot apices of plants induced for 72 hours in continuous light. The first two flower primordia initiated (f1, f2) are indicated in C, and the first four flower primordia (f1*-f4) are indicated in D. The two leaf/paraclade primordia (arrowheads) labeled in C correspond approximately to the third and fourth flower primordium (f3, f4) in D. The ridges which will develop into sepal primordia are not yet evident on the adaxial side of the first flower primordium (f1*) on the apex induced for 72 hours in FR conditions (D), although they are present on the adaxial side of the second flower primordium (f2). Thus, it is likely that this primordium (f1*) is actually a chimeric shoot, i.e., part flower and part flowering shoot (paraclade). (E,F) Shoot apices of plants induced for 96 hours in continuous light. The first two flowers are labeled (f1, f2). In E, the last leaf primordium (arrowhead) and its associated paraclade primordium (asterisk) are indicated. In F, a chimeric shoot that was initiated prior to the initiation of the first two flowers (f1, f2) is apparent. Two sepals (s) cover most of the chimeric shoot. A secondary flower, which differentiated from the chimeric shoot meristem is also evident (asterisk), confirming the identity of the chimeric shoot. Bars: (A) 60 µm; (B) 75 µm; (C,D) 100 µm; (E,F) 145 µm.

time at which flower primordia were first initiated, as there is no known marker for distinguishing very young flower primordia from very young leaf primordia in *Arabidopsis* (Hempel and Feldman, 1995).

Prior to the start of photoinduction only leaf primordia, which contain a region with lateral shoot (paraclade) potential, were seen (Fig. 3A). Flower primordia became morphologically distinguishable from leaf/paraclade primordia 2 days after the start of photoinduction on FR- (Fig. 3B), but not on R-treated plants. After 3 days, plants in both treatments had produced many flower primordia (Fig. 3C,D).

In a pair of representative apices, two young leaf/paraclade primordia are evident at the periphery of the R-treated apex (Fig. 3C). The corresponding primordia on the FR-treated apex, which are at roughly the same stage of primordium development, are flower primordia (Fig. 3D). This morphological difference (shown graphically in Fig. 2) is consistent with the finding that there were also fewer primary-axis leaves on the FR continuous-light control plants (Fig. 4). Four days after the start of photoinduction, the oldest flowers on the FR-treated apices (Fig. 3F) were at a developmentally more advanced stage than the oldest flowers on the R-treated apices (Fig. 3E). Chimeric shoots, when present, were clearly visible at this time (Fig. 3F).

Under FR conditions, 4 hours of light extension (photoinduction) was sufficient for floral determination, and 12 hours of FR light was sufficient to induce the initiation of the first flower at a time equal to the continuous-light control (Fig. 4). In contrast, R-induced plants were only determined after 16
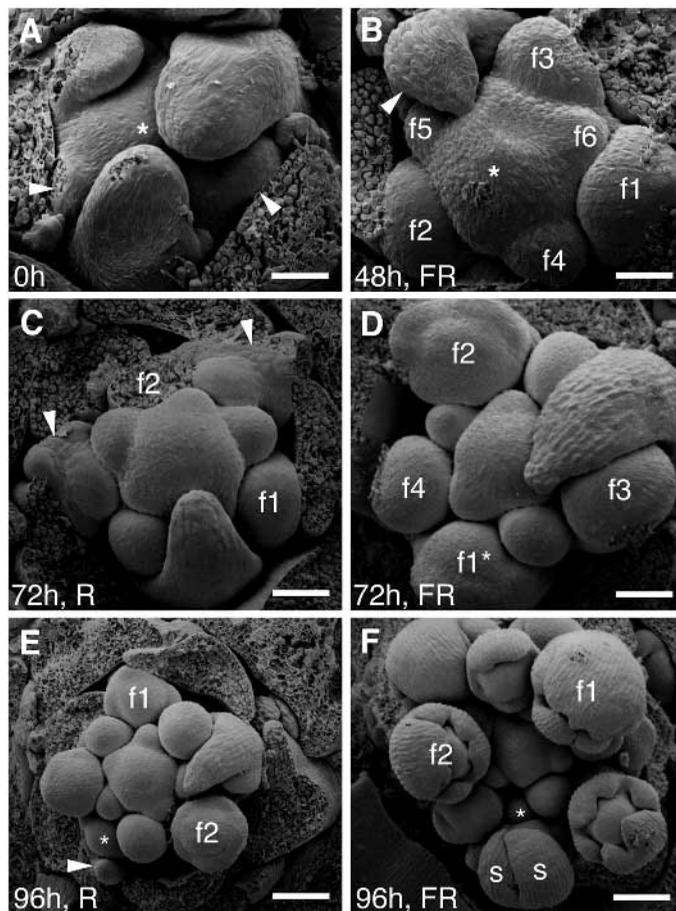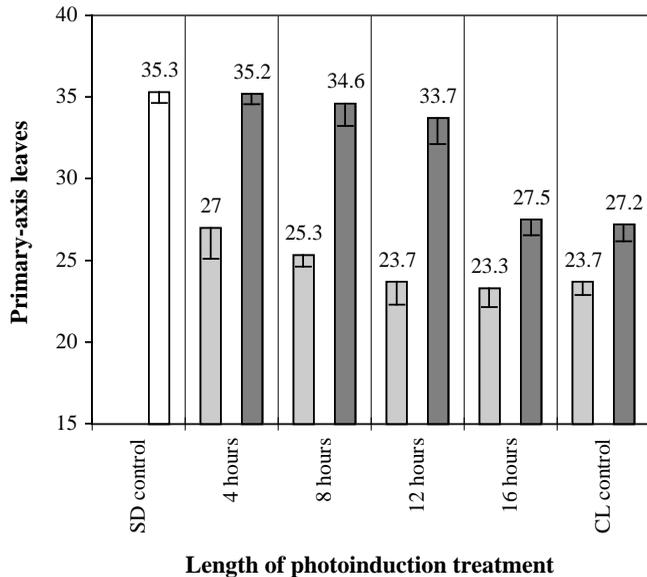
Fig. 4. Determination in R and FR light. Bar chart indicating total primary-axis leaves formed when 25-day old plants were photoinduced in R conditions (dark gray bars) or FR conditions (light gray bars) for 4, 8, 12, or 16 hours (n = 10/treatment). Continuous-light controls were induced in R or FR light after 25 short days. Each treatment population was placed in continuous FR light 10 days after the primary induction treatments (see Fig. 1), along with the short-day control (white bar). Population means and standard deviations of the mean are shown. For clarity, only single-sided error bars (extending below the mean) are included. Florally determined populations are those in which the leaf number equals the appropriate continuous-light control. Undetermined populations are those in which the leaf number is equal to that of the short-day control.

hours of photoinduction (Fig. 4). A comparison of the two continuous-light controls (FR versus R) showed that an average of 3.5 fewer leaves were formed on the FR-induced plants (Fig. 4). This difference indicates that the FR treatment caused leaf production to cease sooner following the start of continuous-light induction.

Photoinduced expression of *LFY, AGL8* and *AP1*

To analyze the transcriptional regulation of *AGL8, LFY* and *AP1* during the photoinduction of flowering, we assayed the activity of *AGL8::GUS, LFY::GUS* and *AP1::GUS* reporters in apices of photoinduced plants by X-gluc staining (Figs 5, 6). These analyses were performed with the same population of plants used in the floral-determination assays.

We did not detect any expression of *AP1::GUS* or *AGL8::GUS* in 5-, 10-, 15-, 20- or 25-day old plants grown under short days (8 hours light/16 hours dark; R conditions). In contrast, *LFY::GUS* activity was readily detected in the shoot apices of young vegetative plants, even at the 5-day time-point, when *Arabidopsis* is insensitive to photoinductive signals (Bradley et al., 1997). Subsequently, there was a dampening of *LFY::GUS* activity, and only low levels were observed in 25-day old plants, at the start of the photoinduction treatments (Fig. 5A-C). This is in contrast to results obtained by Blázquez and colleagues (1997), who report a gradual rise in *LFY::GUS* activity throughout vegetative devel-

opment. The differences between the two experiments could be due to the different ecotypes used (Nossen versus Columbia and Landsberg *erecta*) and/or in the length of the short-day light periods (8 versus 10 hours). They may also be explained by differences in light quality, as light with a very high red-to-far-red ratio, which delays flowering in short days (F. Hempel, unpublished results), was used during the pre-induction growth period in the experiments reported here.

Both *LFY::GUS* and *AGL8::GUS* activity increased markedly within the first 4 hours of photoinduction (compare Fig. 5C,D with Fig. 5E-G), both in the shoot meristem proper and in young leaf/paraclade primordia. In the R-treated plants, we detected a transient decrease of *LFY::GUS* activity after 12 hours (compare Fig. 5E,K). After 16 hours of R- or FR-induction, however, *LFY::GUS* activity had increased substantially, and continued to increase further, with the highest levels of activity being seen in young flower primordia after 3 days (not shown). The overall pattern of *AGL8::GUS* activity, within the first day of photoinduction, was similar to that of *LFY::GUS* (Fig. 5). As with *LFY::GUS*, an increase in *AGL8::GUS* activity preceded floral determination in the R treatment (Fig. 5).

In contrast to *LFY::GUS* and *AGL8::GUS*, activity of *AP1::GUS* was not detected until 16 hours into the photoinduction treatments (Fig. 6A). After 24 hours of FR-induction, low levels of *AP1::GUS* activity were observed in young leaf primordia and at the base of older leaves (Fig. 6B-D). *AP1::GUS* activity continued to increase in young leaves, and eventually in flower primordia (Fig. 6D). The induction of *AP1::GUS* activity occurred more rapidly under FR conditions, and high levels of activity in flower primordia were first detected after 2 days under FR conditions (Fig. 6D).

In a pair of corollary experiments, we compared in situ hybridization of *LFY, AGL8* and *AP1* RNA to GUS activity to assess qualitatively the fidelity of reporter gene expression patterns (Figs 6E,F and 7). Photoinduced expression of *LFY, AGL8* and *AP1* RNA was analyzed in an experiment where Landsberg *erecta* plants grown in short days were induced to flower by transfer to continuous light (Fig. 7). While *LFY* RNA was present in vegetative apices, its levels increased further during photoinduction (Fig. 7). After 3 days, expression of *LFY* RNA was highest in flower primordia, similar to what has been observed in mature flowering apices (Weigel et al., 1992).

AGL8 RNA was also expressed throughout the shoot apex during the 2 days following the start of photoinduction (Fig. 7). After 3 days, however, relatively high levels of expression were limited to the regions of the inflorescence meristem surrounding the flower primordia, again similar to what has been observed in mature flowering apices (Mandel and Yanofsky, 1995a).

In contrast to *LFY* and *AGL8*, no *AP1* RNA was detected in the shoot apex at the start of photoinduction. After the start of induction, low levels of *AP1* RNA were detected at the base of leaf primordia, with high levels being limited to flower primordia, similar to what has been reported for mature flowering apices (Fig. 7) (Mandel et al., 1992). Overall, the expression patterns of *LFY, AGL8* and *AP1* RNA closely resembled those observed for the corresponding *GUS* fusions in the parallel experiment, and the timing of expression in the two experiments was also qualitatively equivalent. For example, high levels of *AP1* expression was detected in the

Fig. 5. Photoinduction of *LFY::GUS* and *AGL8::GUS* activity. All apices were photographed under dark-field illumination. The X-gluc breakdown product, which appears blue under bright-field illumination, appears pink (in tissue with relatively low levels of activity) or blue (in tissues with higher levels of activity) under darkfield illumination. Regions with no GUS activity appear white or light green. (A) *LFY::GUS* expression in a 5-day old vegetative shoot apex. Expression is evident in leaf primordia (arrows), in the apical meristem (asterisk) and in tissues within the apex on the periphery of the meristem. (B) *LFY::GUS* expression in a 15-day old vegetative shoot apex. Highest expression is observed in young leaf primordia (arrow). Asterisk denotes apical meristem. (C,D) *LFY::GUS* and *AGL8::GUS* activity in 25-day old vegetative shoot apices (at the start of photoinduction). Low levels of expression in the *LFY::GUS* apices are apparent in the leaf primordia and at the intersection of the provascular tissue of the leaf primordia and the provascular tissue of the apical meristem (arrows). (E,H,K,N) *LFY::GUS* expression in plants induced with continuous R light. Expression is evident throughout the shoot apex, in the leaf primordia (arrows, E) and in the apical meristem (asterisk, E). (F,I,L,O) *LFY::GUS* expression in plants induced with continuous FR light. (G,J,M,P) *AGL8::GUS* expression in plants induced with continuous R light. Note that *AGL8::GUS* expression in plants induced with continuous FR light were very similar (not shown). For plates E-P, time exposed to photoinductive light is shown in the left margin. All apices were photographed at the same magnification. Bar, 125 μm.
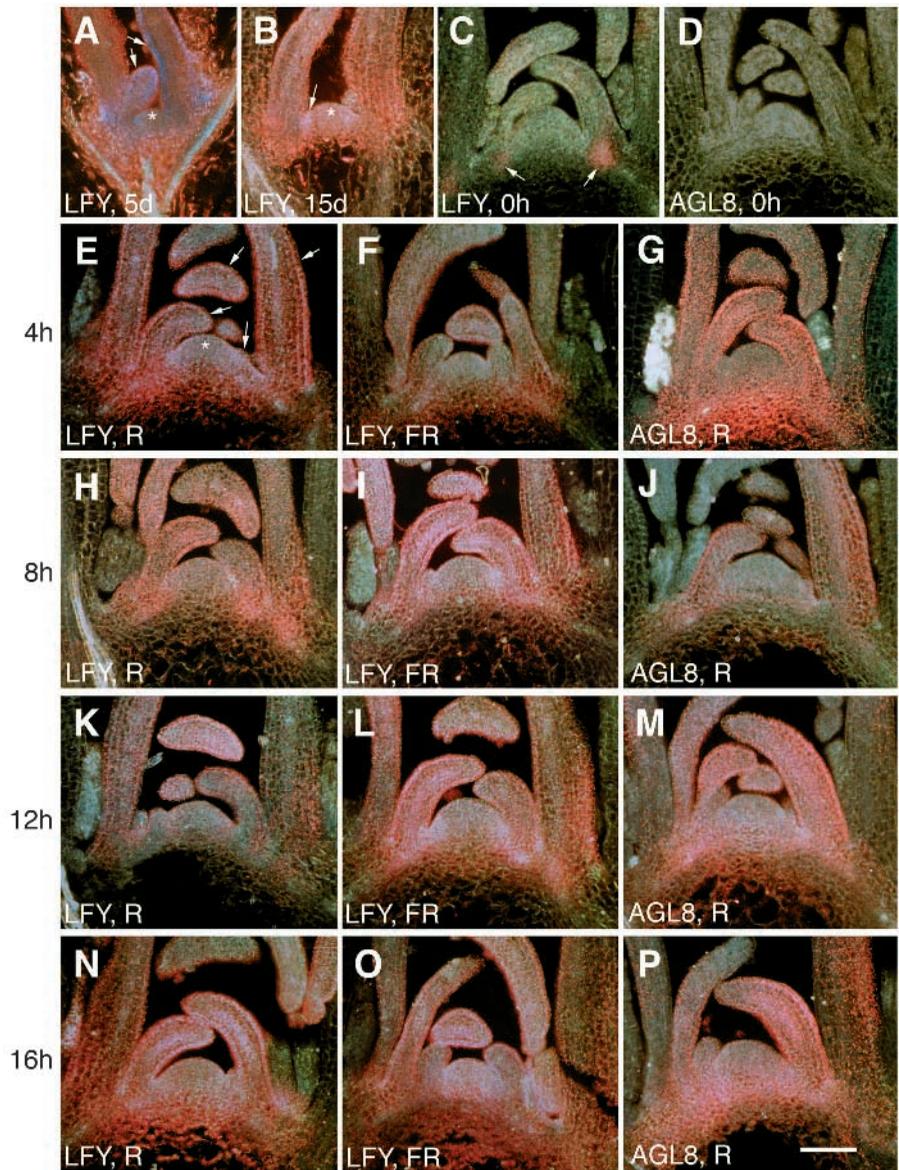
flower primordia after 72 hours of photoinduction in both cases (Figs 6F and 7). The only noticeable difference in the expression patterns was that *AP1::GUS* activity, but not *AP1* RNA, was evident in the young leaf primordia at the 48-hour timepoint (compare Figs 6E and 7).

DISCUSSION

Photoinduction of floral determination and flower initiation

We have shown that the Nossen ecotype of *Arabidopsis*, like the Columbia ecotype (Corbesier et al., 1996), can be induced to flowering by one long day provided the red-to-far-red ratio is sufficiently low. Under red-enriched (R) conditions, floral determination required a total of 20-24 hours of continuous R light, which is more than one 16-hour long-day. In contrast, adding 4 hours of far-red-enriched (FR) light to the end of an 8-hour day of red-enriched light was sufficient for floral deter-

mination. The greater effectiveness of the FR treatment compared to the R treatment occurred despite a considerably lower total irradiance, consistent with previous reports that far-red light is an effective promoter of flowering in *Arabidopsis* (Martínez-Zapater and Somerville, 1990; Goto et al., 1991; Bagnall, 1993; Lee and Amasino, 1995).

Control plants induced with continuous FR-light had fewer leaves than plants that received the briefest FR treatment, of only 4 hours. Similarly, floral determination occurred sooner in control plants that were moved permanently to continuous FR conditions compared to those placed permanently in continuous R conditions. These differences can be explained in one of two ways. One possibility is that there is a conversion of the youngest existing primordia into flower primordia when floral induction signals are sufficiently strong. This would suggest that the fate of the emerging primordia or anlagen is plastic until a certain stage, and that even primordia that have already adopted a bias towards leaf/paraclade fate will assume a floral fate if the inductive signal is potent enough (e.g., in the

Fig. 6. Photoinduction of *AP1::GUS* activity. (A) Very faint *AP1::GUS* expression (arrowhead) 16 hours into FR induction treatment. (B,C) *AP1::GUS* expression 24 hours into FR induction treatment. Expression is evident at the bases of the older leaves (arrowhead) and in leaf primordia (arrows). A-C were photographed under dark-field illumination. (D) *AP1::GUS* expression 24, 36 and 48 hours into photoinduction treatments (R and FR). Arrows indicate leaf primordia on the same shoot apex shown in B and C. Arrowhead indicates a flowering apex with high levels of *AP1::GUS* expression in the young flower primordia. Shoot apices in D were bisected before GUS staining and were photographed under a dissecting microscope. (E,F): *AP1::GUS* expression in young primordia (bright field, Nomarski optics). Plants in E,F were induced with light conditions equivalent to those used in the characterization of RNA expression (Fig. 7). Arrows indicate expression (blue) in leaf primordia of a plant photoinduced for 48 hours. Asterisks denote flower primordia. After 72 hours of photoinduction, *AP1::GUS* expression is highest in the initiated flower primordia.
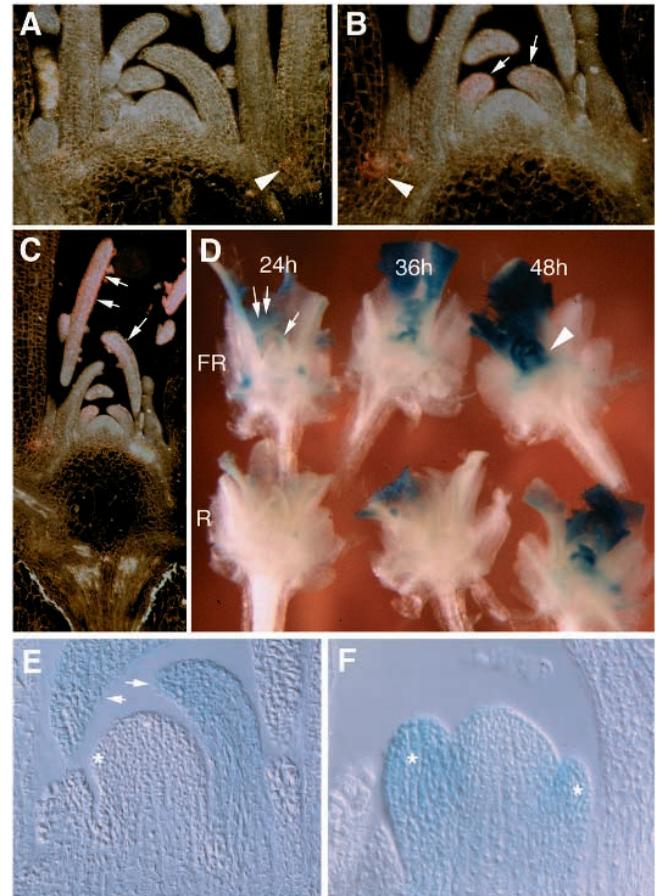
continuous FR treatment). This first explanation is consistent with evidence that in many plants, including *Arabidopsis*, primordium fate is specified progressively during development (Battey and Lyndon, 1990; Bradley, et al., 1996; Hempel, 1996). Alternatively, the production of a small number of leaves may occur after the start of relatively weak inductive conditions (e.g., in the 4 hour FR treatment and in the continuous R treatment).

Expression of floral regulatory genes during photoinduction

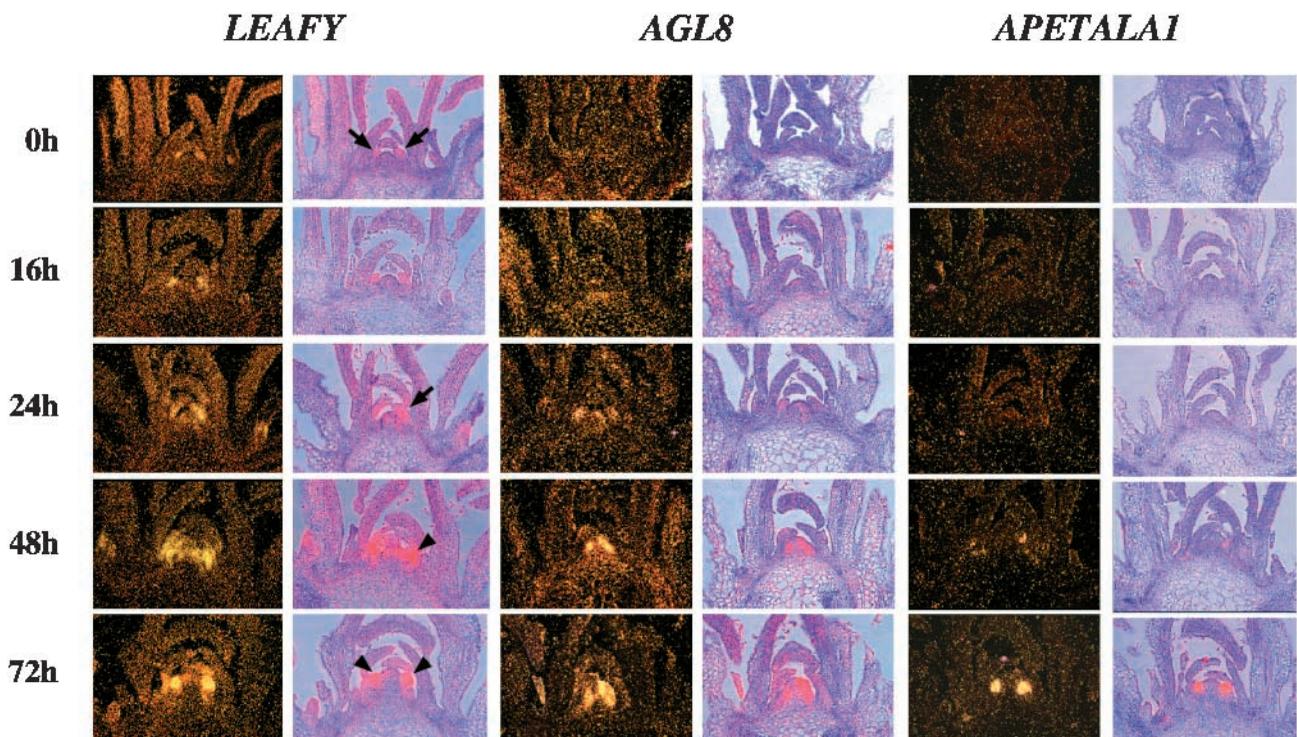The higher effectiveness of the FR treatment, versus the R

Fig. 7. Photoinduction of *LFY*, *AGL8* and *AP1* RNA expression. Longitudinal sections of representative shoot apices of plants induced in continuous light (see Materials and Methods). Time elapsed from the beginning of the induction treatment is indicated in the left margin. Pairs of dark-field (left) and bright-field (right) images are shown for each apex. Arrows indicate leaf primordia and arrowheads indicate flower primordia. Expression in dark-field illumination is shown in yellow; expression in bright-field illumination is in red.

treatment, in promoting a rapid switch from the production of leaf/paraclade to flower primordia was not paralleled by pronounced differences in *AGL8::GUS* and *LFY::GUS* activity profiles. Accordingly, while the increase in *LFY::GUS* and *AGL8::GUS* activity was concurrent with floral determination in the FR treatment, the initial increase in *LFY::GUS* and *AGL8::GUS* activity in the R treatment preceded floral determination by 12 hours. A subsequent decrease in *LFY::GUS* activity in the R treatment was clearly evident after 12 hours of continuous photoinduction, suggesting a potential role for circadian rhythms in the regulation of *LFY.*

The unexpected lack of correlation between specific levels of *LFY::GUS* and *AGL8::GUS* activity and floral determination may indicate that while FR and R treatments are similarly effective in inducing *LFY* and *AGL8*, the R treatment was less effective in promoting the competence to respond to these floral regulators. Recent analyses have demonstrated that in addition to absolute *LFY* levels, other 'competence' factors modulate responses to *LFY* in the apex (Weigel and Nilsson, 1995; Blázquez et al., 1997). In this context, the slight decrease in *LFY::GUS* activity after 12 hours of the R, but not the FR treatment, suggests that one aspect of competence is the ability to maintain levels of *LFY* expression after an early acute response.

Additionally, since we assayed for determination at the whole-plant level, it is possible that the first changes which induced 'determination' in our experiments occurred in the leaves (Zeevaart, 1958; Chailakhyan, 1968). If this is the case, the level of *LFY* expressed in a shoot apex, even shortly after determination has occurred, need not be sufficient for the production of flowers. The low levels of *LFY::GUS* evident in the apex around the time of determination, in our experiments, may simply indicate that although the leaves were determined to send signals sufficient to induce flowering (and floral regulator function), the signals had not yet arrived in full. This explanation fits with experiments on *Lolium temulentum* and *Ipomoea nil* which indicate that determining changes in the leaves precede those in the shoot apex by a few hours (Larkin et al., 1990; McDaniel et al., 1991).

Diffuse patterns of *LFY::GUS* and *AGL8::GUS* were seen during the first 2 days of photoinduction, and early *AP1::GUS* expression was also somewhat diffuse and not strictly localized to flower primordia. Likewise, the expression of *LFY*, *AGL8* and *AP1* RNAs was relatively diffuse during the first 2 days of photoinduction, and qualitatively similar to that of the corresponding reporter constructs. These initially diffuse expression patterns might reflect that upstream regulators of flower-meristem-identity genes are not strictly localized to emerging floral primordia, but that once floral induction has taken place, subsequent interactions among flower-meristem-identity genes are required to sharpen their expression patterns, similar to that observed in other developing primordia such as the *Drosophila* wing (Rulifson et al., 1996).

In these experiments, *AP1::GUS* activity was a sensitive marker for floral determination in both FR and R conditions. Although *AP1::GUS* was expressed when flower primordia were still morphologically indistinguishable from leaf primordia, we detected *AP1::GUS* activity only after floral determination. Thus, our results concur with a recent report indicating that *LFY* expression precedes *AP1* expression when flowering is induced photoperiodically, as well as when it is induced by ectopic expression of the flower-promoting gene *CONSTANS* (Simon et al., 1996).

Quantitative aspects of floral induction

The photoinduction of flowering involves complex interactions between the leaves and the shoot apex. Leaves perceive both photoperiod and light quality (Knott, 1934; Bernier et al., 1993) and send signals to the shoot apex, which is the site of flower production. Floral induction signals from the leaves and other regions of the plant (McDaniel et al., 1992; Kinet et al., 1993), are integrated at the shoot apex, and in sufficient quantity, they induce the initiation of flowers and the expression of flowering genes.

The specific molecular processes which commit an *Arabidopsis* plant to flower are yet to be defined, and our experiments do not resolve the question of whether floral determination is regulated in the leaves or at the shoot apex. However, our results show that plants that are developing a flowering bias, as indicated by transient increases in *LFY::GUS* and *AGL8::GUS* expression, can remain vegetative if returned to non-inductive conditions. This indicates that flower specification is a quantitative process both with respect to the perception of flower-promoting light signals in leaves and to the activity of floral regulatory genes at the shoot apex (McDaniel et al., 1991; Bowman et al., 1993; Schultz and Haughn, 1993; Bradley et al., 1996; Blázquez et al., 1997).

REFERENCES

Bagnall, D. J. (1993). Light quality and vernalization interact in controlling late flowering in *Arabidopsis* ecotypes and mutants. *Ann. Bot.* **71**, 75-83.
Battey, N. H. and Lyndon, R. F. (1990) Reversion of flowering. *Bot. Rev.* **56**, 162-189.
Bernier, G., Havelange, A., Houssa, C., Petitjean, A. and Lejeune, P. (1993). Physiological signals that induce flowering. *Plant Cell* **5**, 1147-1155.
Bernier, G., Kinet, J.-M. and Sachs, R. M. (1981). *The Physiology of Flowering,* Volume I. CRC Press, Boca Raton.
Blázquez, M., Soowal, L., Lee, I. and Weigel, D. (1997). *LEAFY* expression and flower initiation in *Arabidopsis. Development* **124**, 3835-3844.
Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M. and Smyth, D. R. (1993). Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development* **119**, 721-743.
Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., and Coen, E. (1997). Inflorescence commitment and architecture in *Arabidopsis. Science* **275**, 80-83.
Bradley, D., Vincent, C., Carpenter, R., and Coen, E. (1996). Pathways for inflorescence and floral induction in *Antirrhinum. Development* **122**, 1535-1544.
Chailakhyan, M. K. (1968). Internal factors of plant flowering. *Ann. Rev. Plant. Physiol.* **19**, 1-36.
Corbesier, L., Gadisseur, I., Silvestre, G., Jacqmard, A. and Bernier, G. (1996). Design in *Arabidopsis thaliana* of a synchronous system of floral induction by one long day. *Plant J.* **9**, 947-952.

Drews, G. N., Bowman, J. L. and Meyerowitz, E. M. (1991). Negative regulation of the Arabidopsis homeotic gene *AGAMOUS* by the *APETALA2* product. *Cell* **65**, 991-1002.

Esau, K. (1977). *Anatomy of Seed Plants,* 2nd Edition. John Wiley & Sons, New York.

Garner, W. W. and Allard, H. A. (1920). Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. *J. Agricult. Res.* **18**, 553-606.

Goto, K., Kumagai, T. and Koornneef, M. (1991). Flowering responses to light breaks in photomorphogenic mutants of *Arabidopsis thaliana*, a long-day plant. *Physiol. Plant.* **83**, 209-215.

Hempel, F. D. (1996). The morphology of the transition to flowering in mustards. *Semin. Cell Dev. Biol.* **7,** 391-400.

Hempel, F. D. and Feldman, L. J. (1994). Bi-directional inflorescence development in *Arabidopsis thaliana*: Acropetal initiation of flowers and basipetal initiation of paraclades. *Planta* **192**, 276-286.

Hempel, F. D. and Feldman, L. J. (1995). Specification of chimeric flowering shoots in wild-type *Arabidopsis*. *Plant J.* **8**, 725-731.

Huala, E. and Sussex, I. M. (1993). Determination and cell interactions in reproductive meristems. *Plant Cell* **5**, 1157-1165.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901-3907.

Kinet, J.-M., Lejeune, P. and Bernier, G. (1993). Shoot-root interactions during floral transition: a possible role for cytokinins. *Environ. Exp. Bot.* **33**, 459-469.

King, R. A. and Evans, L. T. (1969). Timing of evocation and development of flowers in *Pharbitis nil*. *Aust. J. Biol. Sci.* **22**, 559-572.

Knott, J. E. (1934). Effect of a localized photoperiod on spinach. *Proc. Amer. Soc. Hort. Sci.* **31**, 152-154.

Koornneef, M., Hanhart, C. J. and van der Veen, J. H. (1991). A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **229**, 57-66.

Larkin, J. C., Felsheim, R. and Das, A. (1990). Floral determination in the terminal bud of the short-day plant *Pharbitis nil*. *Dev. Biol.* **137**, 434-443.

Lee, I. and Amasino, R. M. (1995). Effect of vernalization, photoperiod, and light quality on the flowering phenotype of *Arabidopsis* plants containing the *FRIGIDA* gene. *Plant Physiol.* **108**, 157-162.

Mandel, M. A., Gustafson-Brown, C., Savidge, B. and Yanofsky, M. F. (1992). Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. *Nature* **360**, 273-277.

Mandel, M. A. and Yanofsky, M. F. (1995a). The Arabidopsis *AGL8* MADS box gene is expressed in inflorescence meristems and is negatively regulated by *APETALA1*. *Plant Cell* **7**, 1763-1771.

Mandel, M. A. and Yanofsky, M. F. (1995b). A gene triggering flower development in Arabidopsis. *Nature* **377**, 522-524.

Martínez-Zapater, J. M. and Somerville, C. R. (1990). Effect of light quality and vernalization on late-flowering mutants of *Arabidopsis thaliana*. *Plant Physiol.* **92**, 770-776.

McDaniel, C. N., Singer, S. R. and Smith, S. M. E. (1992). Developmental states associated with the floral transition. *Dev. Biol.* **153**, 59-69.

McDaniel, C. N., King, R. W., and Evans, L. T. (1991). Floral determination and in-vitro floral differentiation in isolated shoot apices of *Lolium temulentum* L. *Planta* **185**, 9-16.

Napp-Zinn, K. (1985). *Arabidopsis thaliana*. In *CRC Handbook of Flowering* H. A. Halevy, editor, pp. 492-503. CRC Press, Boca Raton.

Purugganan, M. D., Rounsley, S. D., Schmidt, R. J. and Yanofsky, M. F. (1995). Molecular evolution of flower development: Diversification of the plant MADS-box regulatory gene family. *Genetics* **140**, 345-356.

Rulifson, E. J., Micchelli, C. A., Axelrod, J. D., Perrimon, N. and Blair, S. S. (1996). *wingless* refines its own expression domain on the *Drosophila* wing margin. *Nature* **384**, 72-74.

Simon, R., Igeño, M. I. and Coupland, G. (1996). Activation of floral meristem identity genes in *Arabidopsis*. *Nature* **384,** 59-62.

Schultz, E. A. and Haughn, G. W. (1993). Genetic analysis of the floral initiation process (FLIP) in *Arabidopsis*. *Development* **119**, 745-765.

Thomas, B. and Vince-Prue, D. (1997). *Photoperiodism in Plants*. Academic Press, New York.

Valvekens, D., Van Montagu, M. and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. USA* **85**, 5536-5540.

Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F. and Meyerowitz, E. M. (1992). *LEAFY* controls floral meristem identity in Arabidopsis. *Cell* **69**, 843-859.

Weigel, D. and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. *Nature* **377**, 495-500.

Yanofsky, M. (1995). Floral meristems to floral organs: genes controlling early events in *Arabidopsis* flower development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 167-188.

Zeevaart, J. A. D. (1958). Flower formation as studied by grafting. *Meded. Langbouwhogesch. Wageningen* **53**, 1-88.