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# Early Changes in Gene Expression during the Transition from Vegetative to Generative Growth in the Long-Day Plant *Sinapis alba*

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**Changes in gene expression during flower formation were studied in the long-day plant *Sinapis alba*. The day length dependence was exploited to synchronize flower formation in a large population of mustard plants. After an inductive light treatment, apices were harvested after different lengths of time, and changes in gene expression were analyzed. Two major groups of genes were identified whose expression was affected during flower formation. Transcripts of the first group (group I) were present at low concentration in the apex of noninduced plants. They began to accumulate strongly after the end of the inductive light period. They reached a maximum 2 days to 10 days after flower induction and then declined slowly. Transcripts of the second group of genes (group II) could be detected for the first time 10 days after flower induction. Within a very short time, these transcripts accumulated dramatically and reached a maximum 15 days after flower induction before beginning to decline. They dropped beyond the limit of detection before the flower reached maturity.**

## INTRODUCTION

In many higher plant species, flower formation can be induced by environmental signals such as temperature and day length. In plants that flower in response to an appropriate photoperiod, it is well established that the day length signal is perceived in leaves. An unknown signal is transmitted from leaves to the meristems, which are then converted to floral function (Bernier, 1988). The molecular and physiological processes that occur at the meristem in response to the arrival of the floral stimulus are not well understood. It is generally accepted that during flower formation new genes are expressed that are not active in the vegetative plant (Drews and Goldberg, 1989). It is not obvious, however, when such changes in the expression of genes should first be expected.

In the present work, we studied flower formation in the long-day plant *Sinapis alba*. Flower formation in mustard plants was induced by extending the daily light period from 8 hr to 16 hr. This light dependence enabled us to synchronize large populations of mustard plants and to analyze changes in gene expression during the transition from the vegetative to the flowering state of the apex. Two major groups of genes were distinguished whose expression was affected at different stages of flower formation. Transcripts of the first group of genes (group I) were present already in the vegetative apex but their concentration increased approximately fivefold to 10-fold shortly after the floral stimulus had reached the apex. At this early stage of flower formation, floral specific genes could not be detected whose expression was induced de novo by the floral stimulus. However, transcripts of the second group of genes (group II) were not detectable in the vegetative apex. They appeared specifically only in the developing flower at the time when floral organs began to form.

## RESULTS

Mustard plants were grown for 8 weeks under a daily light regime of 8 hr of light/16 hr of darkness. Flower formation was induced by changing the daily length of illumination from 8 hr to 16 hr. Apical buds of the shoots were harvested after different lengths of time and were fixed and embedded as described in Methods. Sections of the samples were examined microscopically, and changes in

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the morphology of the meristematic tissue of the apex during the transition from the vegetative to the reproductive stage were recorded as shown in Figure 1. Two days after the first long-day treatment, there was no obvious change in the appearance of the meristematic tissue. However, 5 days after flower induction, first primordia of flowers were visible. Ten days after the beginning of the long-day treatment, the eldest flower buds reached a length of approximately 1 mm and contained primordia of different floral organs such as sepals, stamens, and pistils. During the next 10 days, the size of these flower buds increased rapidly. Approximately 25 days after the induction of flowering, the eldest flowers of the inflorescence began to open.

In previous studies, changes in the polypeptide composition of the apical bud during flower formation were described (Lyndon et al., 1983). We tried to reproduce these results in two different ways. First, proteins from apical buds of plants that were exposed for 2 days, 5 days, and 10 days to a daily 16-hr light period were pulse labeled, extracted from the tissue, and separated by two-dimensional polyacrylamide gel electrophoresis. More than 300 spots of radioactively labeled polypeptides could be resolved (results not shown). Even though there were a few minor changes in the intensity of individual spots among different samples, none of these changes was associated with a particular developmental stage of the apex. Similarly, when poly(A)-containing RNA was isolated from apical buds and translated in vitro in a cell-free protein synthesizing wheat germ extract, no obvious changes in the two-dimensional pattern of in vitro products were detectable during the first 10 days after flower induction (results not shown). Both of these results clearly indicate that the methods used so far were not sensitive enough to monitor possible changes in the pattern of gene expression during the early stage of flower formation. Therefore, we used a different and more sensitive approach to detect transcripts of flowering-specific genes that might accumulate during the early stage of flower formation.

Mustard plants were exposed for 2 days, 5 days, and 10 days to a daily 16-hr light period. Apical buds from 2000 plants were harvested for each sample. The poly(A)-containing RNA was isolated and transcribed into cDNA with avian myeloblastosis virus reverse transcriptase, and the RNA was removed by alkaline hydrolysis. The resulting single-stranded cDNA was incubated with a 100-fold excess of poly(A)-containing RNA from young leaves of noninduced control plants and was hybridized to an $R_0t$ value of 1000. The mixture was applied to a hydroxylapatite column and the single-stranded cDNA was separated from the double-stranded cDNA-mRNA hybrids by HPLC chromatography. More than 95% of the cDNA was recovered in the fraction of the double-stranded hybrid molecules. The fraction of single-stranded cDNA should be enriched in apex-specific cDNAs. After the completion of the second strand, these cDNAs were cloned into phage λgt10. These selected cDNAs gave rise to $10^5$ recombinant phage, which should be equivalent to approximately $2 \times 10^6$ recombinant phage before the enrichment step.

The cDNA clones were selected from the λgt10 library by differential screening with $^{32}$P-labeled cDNA. cDNAs derived from the poly(A)-containing RNA of apices of induced or noninduced plants were subjected to the same enrichment procedure as described above. Because the final amount of cDNA that could be recovered after the HPLC chromatography step was less than 50 ng, it was crucial for the preparation of hybridization probes to optimize the labeling procedure. We routinely used probes with a specific radioactivity of approximately $10^9$ cpm/μg DNA. Forty-six and 32 phage were selected out of $10^5$ recombinant phage of the enriched cDNA libraries of 2-day-induced and 5-day-induced mustard plants by differential screening. From the enriched cDNA library of 10-day-induced plants, $2 \times 10^4$ recombinant phage were plated out and 52 recombinant phage were identified by differential screening as putative flowering-specific cDNA clones. From each of these selected recombinant phage, the cDNA insert was subcloned in Bluescribe plasmids.

The identity of putative early flowering-specific cDNAs was tested by dot blot hybridization, as shown in Figure 2. Poly(A)-containing RNA was isolated from apical buds of plants that were either grown vegetatively under a daily 8-hr light/16-hr dark regime or were exposed to a daily 16-hr light period for 2 days, 5 days, 10 days, 15 days, and 20 days. As a control, poly(A)-containing RNA of young leaves of noninduced or induced plants and from mature flowers was included. Equal amounts of RNA were blotted onto stripes of Hybond N membranes and hybridized with $^{32}$P-labeled cDNA inserts of putative flowering-specific cDNA clones. Among the recombinant phage selected from the cDNA libraries of 2-day-induced or 5-day-induced plants, six cDNAs represented transcripts that were specifically expressed in the apex but not in the leaf (group I cDNAs). These cDNAs hybridized to transcripts that were present in apices of both noninduced and induced plants but whose relative concentration increased significantly when plants were exposed to a daily 16-hr light period. These cDNAs were characterized in greater detail. Each cDNA was transcribed in both directions into radioactively labeled sense and antisense RNA. These RNA samples were used as probes for in situ hybridization with sections of apices of 5-day-induced plants. Controls with sense RNA gave only a very weak background reaction with no preferential labeling of a particular tissue (results not shown). Antisense probes of clone pSFD 2.43 hybridized with transcripts in the peripheral cell layers of the receptacle and with transcripts in the cell layers next to the procambial strands of the stem and the leaf primordia and not with the floral meristem, as shown in Figure 3B. When antisense transcripts of clone pSFD 5.04 were hybridized with sections of the same apex sample, the spatial pattern of hybridization signals was different. Transcripts were

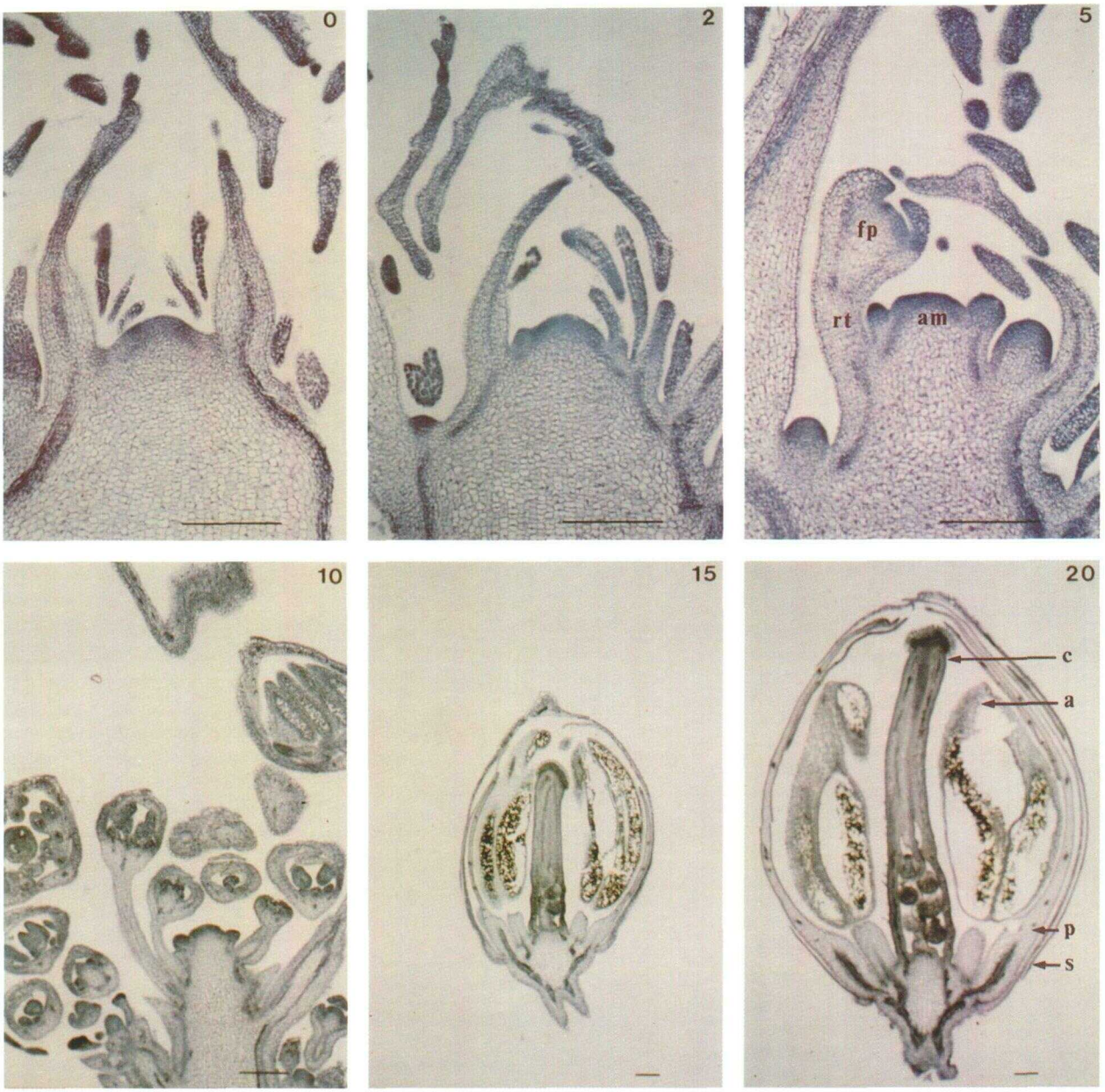


**Figure 1.** Transformation of Vegetative into Reproductive Buds.

Mustard plants were grown vegetatively under short-day conditions for 56 days. Flower formation was induced by changing the daily length of illumination from 8 hr to 16 hr. Apical buds from plants that were exposed for 2 days, 5 days, 10 days, 15 days, and 20 days to a long-day treatment were fixed and embedded. Longitudinal sections of these buds were prepared and stained with toluidine blue. (0) Vegetative apical bud. (2) After 2 long-day periods no morphological changes are obvious, whereas after 5 long days (5) a flower primordia (fp) and a receptacle (rt) are visible, and at the periphery of the apical meristem (am), other floral primordia are formed. (10) At the 10 day stage, flower primordia and flower buds of different stages are visible. Fifteen (15) and 20 (20) days after the beginning of the long-day treatment, sepals (s), petals (p), anthers (a), and carpels (c) are developed. Bar = 250 $\mu$m.

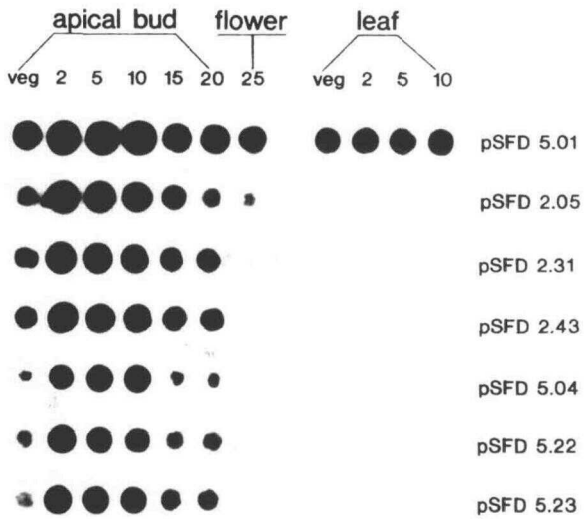


**Figure 2.** Regulation of Gene Expression during the Transition of the Vegetative to the Reproductive State of Apical Buds.

Induction of flower formation was synchronized in a large population of vegetatively growing mustard plants by long-day treatment. Group I cDNAs were selected from two cDNA libraries that were prepared from transcripts of plants 2 days or 5 days after flower induction. Changes in gene expression during flower development were analyzed in plants that were kept under short-day conditions (veg) or were exposed for 2 days, 5 days, 10 days, 15 days, 20 days, and 25 days to a daily 16-hr light period. Equal amounts of poly(A)-containing RNA isolated from apical buds, mature flowers, and leaves were dotted onto Hybond N filters. Hybridization was done with $^{32}$P-labeled cDNAs that represent transcripts that were expressed in apical buds, flowers, and leaves or were present only in the apical bud.

found almost exclusively in the peripheral zone of the apical meristems and within the floral buds, as shown in Figure 3D.

cDNAs of clones pSFD 2.43 and pSFD 5.04 represent mRNAs whose concentrations increase fivefold to 10-fold in the apex during the early stages of flower development of mustard plants. They were selected from cDNA libraries of plants that had been exposed for 2 days or 5 days to a long-day treatment. Similar cDNAs were not found in the third cDNA library of plants that had been exposed for 10 days to a daily 16-hr light period. Several cDNAs were selected from this third library (group II cDNAs), none of which represented transcripts that were present during the early stage of flower development. Transcripts were detected by these cDNAs only 10 days after the beginning of flower induction. Within the next few days, the concentration of these transcripts increased drastically and reached its maximum approximately 15 days after flower induction, as shown in Figure 4. Within the next 5 days, the concentration of these transcripts declined again. When the flower reached maturity, these transcripts were no longer detectable. Some of the group II cDNAs cross-hybridized under high stringency and, thus, seemed to represent identical or closely related transcripts. The remaining seven cDNAs were distinct from each other. In situ hybridization experiments demonstrate that all of these cDNAs represent mRNAs that are localized within

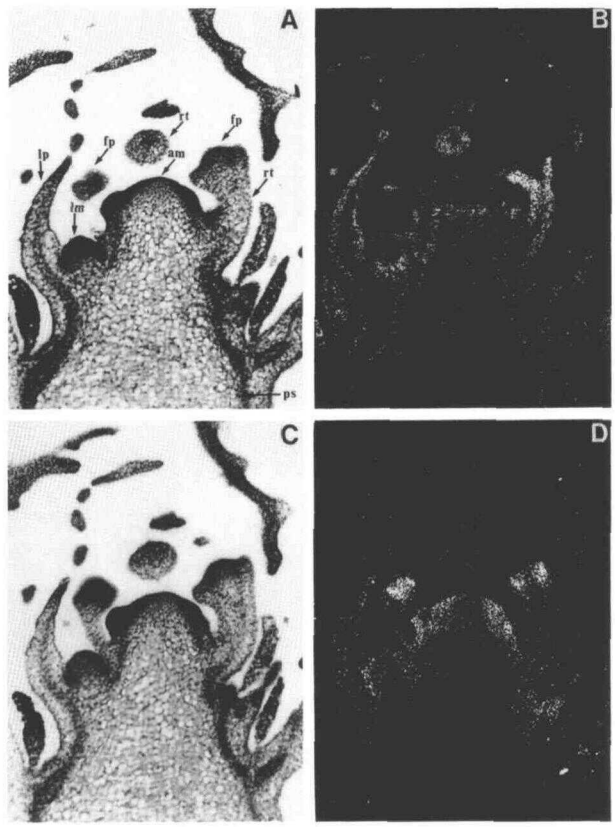


**Figure 3.** In Situ Hybridization of Group I cDNAs in the Apical Bud.

**(A)** Bright-field micrograph of a longitudinal section from an apical bud 5 days after flower induction. Flower primordia (fp) and receptacles (rt) are developed. Axillary to a leaf primordia (lp), a lateral meristem (lm) begins to develop a lateral inflorescence. The procambial strands (ps) in the stem and the leaf primordia are intensely stained.

**(B)** Dark-field micrograph of the in situ hybridization with single-stranded antisense $^{35}$S-labeled RNA probes of the cDNA clone pSFD 2.43. The hybridization signals on the same section as shown under **(A)** are mainly confined to the cortex of the receptacle and the cell layers adjacent to the procambial strands.

**(C)** Bright-field micrograph of another serial section of the same apex as shown under **(A)**.

**(D)** Dark-field micrograph of the same section shown under **(C)** after in situ hybridization with single-stranded antisense $^{35}$S-labeled RNA probes of the cDNA clone pSFD 5.04. The peripheral cell layers of the apical meristem and regions within the floral buds showed hybridization signals.

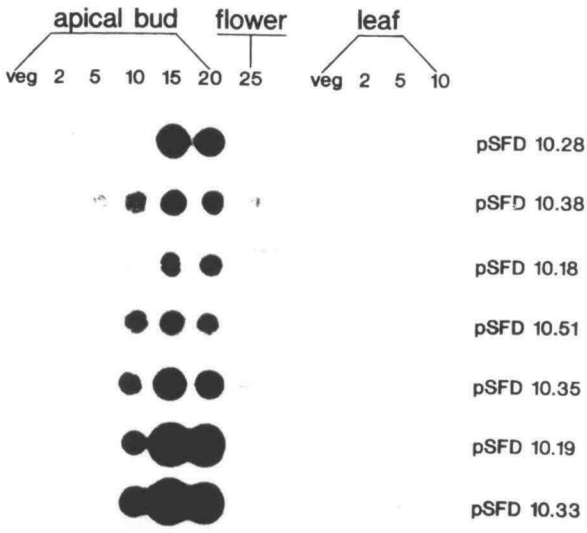


**Figure 4.** Regulation of Gene Expression during the Later Stage of Flower Formation.

Group II cDNAs were selected from a cDNA library in λgt10 that was prepared from transcripts of apical buds 10 days after flower induction. Changes in gene expression during flower development were analyzed in plants that were kept under short-day conditions (veg) or were exposed for 2 days, 5 days, 10 days, 15 days, 20 days, and 25 days to an inductive 16-hr daily light period. Equal amounts of poly(A)-containing RNAs isolated from apical buds, mature flowers, or leaves were dotted onto Hybond N filters. These filters were hybridized with $^{32}$P-labeled cDNAs that represent transcripts that were expressed specifically during the later stage of flower formation but not in the mature flower.

the tapetum. As an example, the in situ hybridization pattern of one of these cDNAs (pSFD 10.20) is shown in Figure 5. These tapetal-specific mRNAs accumulate early in anther development when the tapetum is active and decay when the pollen is mature and the tapetum is dissolved. Thus, these transcripts are not expected to be present in the mature inflorescence.

## DISCUSSION

Flower formation represents one of the most dramatic changes in the development of higher plants. To understand the molecular mechanisms involved in the transition from the vegetative to the reproductive stage, it is necessary to identify genes that are expressed before and/or during the organization of the floral meristem (Drews and Goldberg, 1989). Because there are only a small number of target cells within the apical bud, studies of these early events of flower formation are extremely difficult. Two approaches have been used for this purpose. First, tobacco explants that are capable of flower formation in vitro have been used to identify flower-specific cDNAs (Meeks-Wagner et al., 1989). This in vitro flowering system led to the identification of mRNAs whose concentration increased during floral initiation. However, these mRNAs were also present in mature plant roots and leaves. Thus, the significance of the mRNAs for flower development is not clear yet (Drews and Goldberg, 1989). In our present work, we used a second experimental approach. Flower formation in mustard plants was induced by extending the daily light period from 8 hr to 16 hr (Bodson, 1985). This light dependence of flower formation was exploited to synchronize populations of mustard plants and to obtain

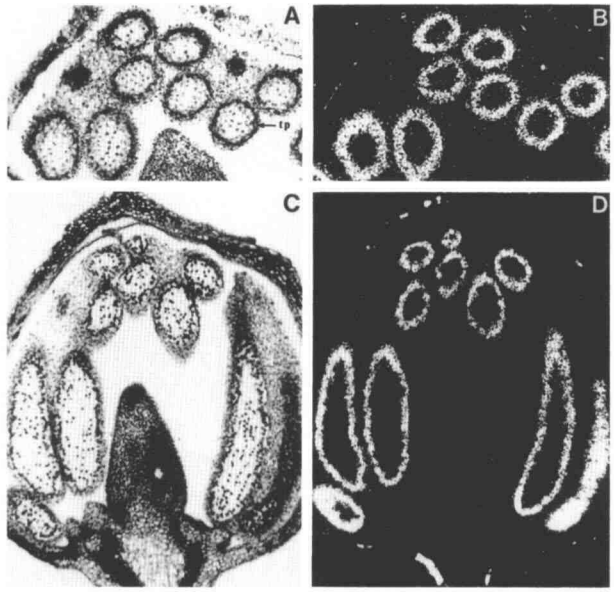


**Figure 5.** In Situ Hybridization of a Group II cDNA in Flower Buds.

The apical buds of a plant 15 days after flower induction were fixed and embedded in paraffin. Sections were hybridized with single-stranded $^{35}$S-labeled antisense RNA probes of the cDNA pSFD 10.20.

**(A)** Bright-field micrograph of a transverse section through anthers. The tapetum (tp) cell layer is intensely stained with toluidine blue.

**(B)** Dark-field micrograph of the section shown under **(A)** after in situ hybridization. Very strong hybridization signals are present only within the cells of the tapetum.

**(C)** Bright-field micrograph of an outermost floral bud from the same inflorescence as the floral bud under **(A)** with a lower magnification.

**(D)** Dark-field micrograph of the same bud as under **(C)** after in situ hybridization. Hybridization signals are only in the tapetum cells. Some small white areas are produced by refractions of light at vascular bundles.

from them large numbers of floral meristems at similar developmental stages.

In previous work with mustard plants, changes in the polypeptide and mRNA compositions of apical buds were described during the initial phase of flower formation that suggested dramatic changes in gene expression during the transition of the shoot meristem from the vegetative to the flowering state (Lyndon et al., 1983). In our work, we were unable to see such changes in gene expression when mRNAs and pulse-labeled polypeptides from apices of noninduced and induced plants were analyzed and compared with each other. Even though some minor changes in the intensity of individual spots could be detected in the pattern of polypeptides that were resolved by two-dimensional polyacrylamide gel electrophoresis, none of these variations was associated with a particular developmental stage during flower formation.

Subtractive hybridization was used successfully for the identification of low abundant transcripts (Davis et al., 1984) and developmentally regulated genes (Zimmermann et al., 1980). In our experiments, more than 95% of the original cDNAs could be separated by subtractive hybridization from cDNAs that were enriched in flowering-specific transcripts. However, even after this enrichment step, only a very small number of cDNAs could be selected by differential screening. Six of these cDNAs represented transcripts that were expressed specifically in the apical bud (group I cDNAs). They could not be detected in young leaves of induced or noninduced plants or in mature flowers. Although these transcripts were detectable already in the apical bud of noninduced plants, in all cases their concentration increased fivefold to 10-fold in response to the long-day treatment (Figure 2).

The earliest stage of floral evocation analyzed in mustard plants was 2 days after the first long-day treatment. Previous studies of mustard plants showed that within 16 hr to 20 hr after the end of the first long-day treatment, the floral stimulus released from the leaves reached the apex (Bernier et al., 1981). Thus, it is likely that the increase in transcript level found in the present work is part of the very early response of the apical meristem to the arrival of the floral stimulus. One of the very first changes observed in the apical meristem of mustard plants after flower evocation is a rapid stimulation of cell division. In the central and peripheral zone of the meristem, mosaics of rapidly cycling and noncycling cells were observed. During flower induction, the cell cycle length was reduced from 86 hr to 32 hr and the proportion of rapidly cycling cells was increased from between 30% and 40% to between 50% and 60%. These changes were detectable as early as 30 hr after flower induction (Gonthier et al., 1987). Even though most of the selected group I cDNAs might be expected to represent mRNAs associated with this early general enhancement of mitotic divisions, results of the in situ hybridization did not support this assumption. The six cDNAs were classified into two subgroups. The cDNA clone pSFD 5.04 was the only member of the first subgroup and represented transcripts that were confined to the peripheral zone of the floral meristems. Transcripts represented by the second subgroup of group I cDNA clones did not accumulate in the apical meristem but were highly concentrated in well-defined zones of peripheral cells at the receptacle and in cells next to the procambial strands.

None of these transcripts appeared to be associated with processes common to all rapidly dividing cells. Transcripts represented by the first subgroup of cDNA clones that were found in the peripheral zone of the meristem did not accumulate in the adjacent central part of the meristem in which cells divide as rapidly as in the peripheral zone (Gonthier et al., 1987). Even though the function of the proteins encoded by the transcripts of the two subgroups is not known yet, they seem to be involved in a more specific aspect of cell division that is confined to particular cell types and may be part of the functional specialization of different cell populations during flower formation.

Several control experiments indicated that the spatial pattern of the group I transcripts reflects their distribution in the intact plant. Hybridization with rRNA probes gave a strong hybridization signal with all cells of the apical tissue without any preferential accumulation of silver grains in one particular cell type. Thus, different parts of the apex must be equally well accessible to the hybridization probe. Sense probes were used routinely as controls in all hybridization experiments and gave no significant hybridization signals of the tissue samples above background level.

Transcripts represented by the group I cDNA clones reached their maximum level 2 days after flower induction and remained at this high level for the next 6 days to 10 days before they slowly declined. At this later stage of flower development, flower buds were clearly visible with distinct primordia of different flower organs such as sepals, stamens, and pistil. This result raises the question of whether or not transcripts of group I genes may also be involved in the formation of these flower structures. Two lines of evidence indicate that group I genes are not involved in the formation of floral organs. First, apices of mustard give rise to many flowers of different age that finally form a complex inflorescence. While the first flowers mature, new flower primordia are formed successively from the apical meristem. For instance, 10 days after flower induction, different stages of floral development are detectable in the short apex ranging from young flower primordia to more mature flower buds with petals, sepals, stamens, and pistil. Our in situ hybridization studies indicated that transcripts of group I genes are confined to the apical bud and to newly formed flower primordia and do not accumulate in more mature flower buds. Second, a large group of cDNAs (group II cDNAs) was selected by differential screening from the cDNA library of 10-day-

induced plants. All of these cDNAs represented transcripts that were found first in the apical bud of plants 10 days after flower induction. These transcripts were not detected at earlier stages of flower formation. During the next 5 days, they reached the maximal concentration and then declined rapidly. In mature flowers, their concentration dropped beyond the limit of detection. The rapid appearance of these transcripts coincided with the formation of floral organs. Thus, it seems likely that these transcripts encode proteins that are involved in the differentiation and formation of different parts of the flower.

This interpretation has been supported by the results of in situ hybridization. All of the selected group II cDNAs represented transcripts that are confined to the tapetum layer. This result was rather surprising because in other studies of flower development, flower-specific transcripts were described that occur also in other floral organs (Goldberg, 1988, Gasser et al., 1989). The results of our present work may indicate that during the formation of floral organs, by far the most dramatic changes at the level of mRNA may occur in the tapetum layer. This tissue becomes active during early stages of anther development and decays rapidly after the maturation of pollen. This transient appearance of the tapetum layer may explain why the tapetum-specific mRNAs are expressed only during a short time period. It is generally assumed that the tapetum layer is primarily involved in the nutrition of the growing pollen grain and in the synthesis of its complex wall. Brassicaceae have evolved the system of sporophytic self-incompatibility in which both alleles of the S-locus of the surrounding sporophytic tissue contribute to the deposition of self-incompatibility factors in the wall of pollen grains (Nasrallah and Nasrallah, 1989). Because white mustard belongs to the group of self-incompatible species of Brassicaceae (Ford and Kay, 1985), it seems possible that one of the tapetum-specific cDNAs may represent transcripts derived from the S-gene(s).

Genes involved in the development of a flower have been divided into two classes according to the function of their products. One class, the regulatory genes, consists of those genes whose primary function is to modulate the activity of other genes. Indirect evidence from genetic studies of homeotic flower mutants clearly indicates that such regulatory genes take part in the control of flower development (Haughn and Somerville, 1988; Komaki et al., 1988; Bowman et al., 1989). Recently, two of these genes were shown to encode proteins closely related to mammalian and yeast transcription factors (Sommer et al., 1990; Yanofsky et al., 1990). The second class of genes involved in development are those whose products form a functional part of the flower. It seems likely that all of the cDNAs studied in the present work represent genes of the latter class.

Our results indicate that within the second class different subgroups of genes may be distinguished whose products are expressed rather specifically at different times and in different tissues during flower formation. One of the most intriguing problems for the future will be the question of how the coordination and interplay of these different subgroups of genes are regulated.

## METHODS

### Plant Material

Mustard plants (*Sinapis alba*) were grown on soil at 20°C in growth chambers. After 56 days under short-day conditions with 8-hr photoperiods, flowering was induced with 16-hr photoperiods.

For RNA and protein isolation, apical buds of vegetative and induced stages were freed of leaf primordia with a pair of tweezers and were frozen in liquid nitrogen. Leaf RNA for the cDNA subtractive hybridization and for the dot-blot analysis was extracted from young leaves 0.5 cm to 2 cm in length.

### Protein Analysis

Proteins of apical buds of 20 plants of one particular developmental stage were labeled in vivo with 5 $\mu$Ci of $^{35}$S-methionine (1 $\mu$g/$\mu$L), which was applied in a 1% Tween solution with a pipette tip. After 4 hr of incubation, the buds were collected and frozen in liquid nitrogen.

Buds were ground under liquid nitrogen and were transferred to the extraction buffer (50 mM Tris-HCl, pH 7.0, 250 mM sodium ascorbate, 1% $\beta$-mercaptoethanol, 5 mM diethyldithiocarbamide, and 5% polyclar AT). The debris was pelleted and the proteins in the supernatant were precipitated with 4 volumes of acetone. The proteins were redissolved and separated on two-dimensional gels according to O'Farrel (1975).

### RNA Isolation

RNA was isolated with some modifications as described by Chirgwin et al. (1979).

The plant material was ground under liquid nitrogen with a mortar and a pestle and homogenized in 4 mL of lysis buffer (4 M guanidinium isothiocyanate, 50 mM Hepes-KOH, pH 7.4, 2% *N*-lauroyl-sarcosin and 1% $\beta$-mercaptoethanol) with an Ultra Turrax (Janke and Kunkel, D7813 Stanfen, F. R. G.). Cell debris and denatured proteins were pelleted and the nucleic acids in the supernatant were precipitated overnight in the presence of 0.75 volumes of ethanol. The pellet was homogenized in 4 mL of Tes (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1% SDS) with the help of a potter, and the homogenate was extracted with phenol/chloroform and chloroform. Total RNA was precipitated overnight with 2 M LiCl at 4°C. Poly(A)-containing RNA was isolated according to Aviv and Leder (1972).

### cDNA Enrichment and Cloning

The first cDNA strand was synthesized by a modification of the procedures of Murray et al. (1983) and Watson and Jackson

(1985). Ten micrograms of poly(A)-containing RNA were reverse transcribed in a 100-μL reaction volume containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM MgCl, 500 μM deoxynucleotide triphosphates, 5 μg of oligo-p(dT)$_{12-18}$, 4 mM sodium pyrophosphate, 10 mM DTT, 100 units of RNasin, 10 μCi of $^{32}$P-dATP, and 100 units of reverse transcriptase (Boehringer). After 1 hr at 42°C, the reaction was stopped with 20 mM EDTA and was extracted with equal volumes of phenol/chloroform and chloroform. The RNA was hydrolyzed with 0.4 M NaOH for 2 hr at 56°C, and the cDNA (2 μg) was then desalted over a Sephadex G-50 column with Tes and precipitated after the addition of poly(A)-containing RNA from young leaves.

The subtractive hybridization (Timberlake, 1980) was carried out with a 100-fold mass excess of leaf RNA (2 μg/μL) in 240 mM phosphate buffer, pH 7.0, 1 mM EDTA, and 0.1% SDS at 68°C in an oven until an $R_0t$ value of 1000 was reached. The $R_0t$ value was calculated according to Van Ness and Hahn (1982).

The single-stranded cDNA (sscDNA) was separated from the cDNA/mRNA hybrids by HPLC chromatography on a Bio-Gel high performance hydroxylapatite column (Bio-Rad) according to the instructions of the supplier. The eluted sscDNA was concentrated by several butanol extractions and was desalted over a Sephadex G-50 column with Tes and ethanol precipitated after addition of 10 μg of glycogen (Boehringer) as a carrier.

The sscDNA was tailed at the 3′ end according to Deng and Wu (1981) with a 50-fold molar excess of dCTP. The reaction was carried out in 20-μL tailing buffer (100 mM potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, and 0.2 mM DTT) with 12 units of terminal transferase (Boehringer) at 30°C for 30 min. The tailed cDNA was phenol and chloroform extracted and ethanol precipitated.

Before the synthesis of the second strand, oligo-pd(G)$_{12-18}$ was annealed to the dC tail according to Land et al. (1981) in 100 mM Hepes-KOH, pH 6.9, 10 mM $MgCl_2$, 70 mM KCl, 1 mM DTT, 250 μM each of all four nucleotides, and 10 μCi of $^{32}$P-dATP. Ten units of Klenow polymerase were added and the synthesis of cDNA was carried out at 16°C. After 1 hr, 2 units of T4 DNA-polymerase were added and the reaction was incubated for 15 min at 37°C. EDTA was added to a final concentration of 25 mM and internal EcoRI sites were blocked with 80 μM of *S*-adenosylmethionine and 40 units of EcoRI methylase during 30 min at 37°C. The reaction mixture was heated to 70°C for 10 min, phenol extracted, and passed over a Sephadex G-50 column as described above, and the cDNA was ethanol precipitated.

EcoRI linkers were ligated in a 10-μL reaction volume with 5 units of T4 ligase at 14°C. After 16 hr, the ligase was heat denatured and the reaction mixture was diluted to 20 μL. Concatemeric linkers were digested with 20 units of EcoRI for 3 hr. After one phenol extraction, the unattached linkers were removed from the cDNA on a Sephadex G-50 column with Tes.

One microgram of λgt10 arms (Protoclone, Madison, WI) was ligated with 20 ng of cDNA in a 10-μL reaction with 1 unit of T4 ligase for 16 hr at 14°C. The cDNA was packaged with Gigapack Gold extracts (Stratagene) and plated with *Escherichia coli* C600 hfl according to Huynh et al. (1985).

### Library Screening

Up to 25,000 recombinant phage were plated on a 23 × 23-cm Nunc-plate. Replica plaque lifts were prepared on Hybond N membranes and were treated as suggested by the manufacturer. The differential screenings were performed with oligolabeled probes (Feinberg and Vogelstein, 1983) that were prepared from subtracted sscDNAs from vegetative and induced developmental stages. With less than 50 ng of sscDNA and 100 μCi of $^{32}$P-dATP as a label, a second strand with a total incorporation of $1 \times 10^8$ cpm was synthesized. Free nucleotides were separated from the cDNA over a Sephadex G-50 column.

The filters were prehybridized for 6 hr in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/mL salmon sperm DNA at 65°C. The hybridization was performed in the same solution with $1 \times 10^7$ cpm/mL labeled cDNA for 36 hr to 60 hr.

Filters were washed twice in 2 × SSC and 0.5 × SSC with 0.1% SDS at 62°C and exposed to Kodak XAR films for 2 days to 4 days.

Positive plaques were purified through a second screening at lower plaque density. Inserts of the purified phage were subcloned into Bluescribe plasmids (Stratagene) by standard methods.

### RNA Dot Blots

For each sample, 1 μg of poly(A)-containing RNA was dotted onto Hybond N membranes. Membrane stripes were hybridized with labeled RNA probes, which were transcribed from the recombinant Bluescribe plasmids with 20 μCi of $^{32}$P-UTP as described by the supplier. Before RNA synthesis, the plasmids were linearized with NarI (T3 polymerase) or BamHI or HindIII (T7 polymerase). Hybridization and post-hybridization conditions were as described above. After exposure on Kodak XAR films, the bound radioactivity of the individual dots was counted in a scintillation counter, and the relative amounts of transcripts were calculated as described by Batschauer and Apel (1984).

### In Situ Hybridization

Apical buds and flower buds were fixed in 2% formaldehyde and 0.1% glutaraldehyde in 100 mM phosphate buffer, pH 7.0, for 20 hr at room temperature. The fixed buds were dehydrated and embedded in paraffin by standard methods. Longitudinal sections (10 μm) of the buds were cut with a rotary microtome with glass knives. The sections were transferred to a drop of water on poly-L-lysine-covered slides. The prehybridization treatments, the hybridization with RNA probes, and the post-hybridization treatments were performed according to Angerer et al. (1987).

Antisense and sense RNA probes were prepared as described above with $^{35}$S-UTP as label and were partially hydrolyzed to an average length of 150 bases.

The slides were developed after 3 days to 10 days of exposure with Kodak D19 developer and were fixed in Agfa G350 fixer. The samples were dehydrated with ethanol transferred to xylene and mounted with DePeX (Serva Fine Biochemicals, Garden City Park, NY). The sections were viewed through a Zeiss Axiophot microscope and pictures were taken on Agfa pan 25 films.

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## REFERENCES

**Angerer, L.M., Cox, K.H., and Angerer, R.C.** (1987). Demonstration of tissue-specific gene expression by in situ hybridization. Methods Enzymol. **152,** 649–661.

**Aviv, H., and Leder, P.** (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymedilic acid-cellulose. Proc. Natl. Acad. Sci. USA **69,** 1408–1412.

**Batschauer, A., and Apel, K.** (1984). An inverse control by phytochrome of the expression of two nuclear genes in barley (*Hordeum vulgare* L.). Eur. J. Biochem. **143,** 593–597.

**Bernier, G.** (1988). The control of floral evocation and morphogenesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39,** 175–219.

**Bernier, G., Kinet, J.M., and Sachs, R.M.** (1981). The Physiology of Flowering, Vol. 1 (Boca Raton: CRC Press).

**Bodson, M.** (1985). *Sinapis alba*. In Handbook of Flowering. Vol. 4, A.H. Halevy, ed (Boca Raton: CRC Press), pp. 336–354.

**Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M.** (1989). Genes directing flower development in *Arabidopsis*. Plant Cell **1,** 37–52.

**Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J.** (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18,** 5294–5299.

**Davis, M.M., Cohen, D.I., Nielsen, E.A., Steinmetz, M., Paul, W.E., and Hood, L.** (1984). Cell-type-specific cDNA probes and the murine I region: The localization and orientation of $A_\alpha^d$. Proc. Natl. Acad. Sci. USA **81,** 2194–2198.

**Deng, G., and Wu, R.** (1981). An improvement procedure for utilizing terminal transferase to add homopolymers to the 3′ termini of DNA. Nucl. Acids Res. **9,** 4173–4188.

**Drews, G.N., and Goldberg, R.B.** (1989). Genetic control of flower development. Trends Genet. **5,** 256–261.

**Feinberg, A.P., and Vogelstein, B.** (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13.

**Ford, M.A., and Kay, Q.O.N.** (1985). The genetics of incompatibility in *Sinapis arvensis* L. Heredity **54,** 99–102.

**Gasser, C.S., Budelier, K.A., Smith, A.G., Shah, D.M., and Fraley, R.T. (1989).** Isolation of tissue-specific cDNAs from tomato pistils. Plant Cell **1,** 15–24.

**Goldberg, R.B.** (1988). Plants: Novel developmental processes. Science **240,** 1460–1467.

**Gonthier, R., Jacqmard, A., and Bernier, G.** (1987). Changes in cell cycle duration and growth fraction in the shoot meristem of *Sinapis* during floral transition. Planta **170,** 55–59.

**Haughn, G.W., and Somerville, C.R. (1988).** Genetic control of morphogenesis in *Arabidopsis*. Dev. Genet. **9,** 73–89.

**Huynh, T.V., Young, R.A., and Davis, R.W.** (1985). Construction and screening of cDNA libraries in λgt10 and λgt11. In DNA Cloning: A Practical Approach, Vol. 1, D.M. Glover, ed (Oxford: IRL Press), pp. 49–78.

**Komaki, M.K., Okada, K., Nishino, E., and Shimura, Y.** (1988). Isolation and characterization of novel mutants of *Arabidopsis thaliana* defective in flower development. Development **104,** 195–203.

**Land, H., Grez, M., Hauser, H., Lindenmaier, W., and Schütz, G.** (1981). 5′-Terminal sequences of eucaryotic mRNA can be cloned with high efficiency. Nucl. Acids Res. **9,** 2251–2266.

**Lyndon, R.F., Jacqmard, A., and Bernier, G.** (1983). Changes in protein composition of the shoot meristem during floral evocation in *Sinapis alba*. Physiol. Plant **59,** 476–480.

**Meeks-Wagner, D.R., Dennis, E.S., Tran Thanh Van, K., and Peacock, W.J.** (1989). Tobacco genes expressed during in vitro floral initiation and their expression during normal plant development. Plant Cell **1,** 25–35.

**Murray, M.G., Hoffman, L.M., and Jarvis, N.P.** (1983). Improved yield of full-length phaseolin cDNA clones by controlling premature anticomplementary DNA synthesis. Plant Mol. Biol. **2,** 75–84.

**Nasrallah, J.B., and Nasrallah, M.E.** (1989). The molecular genetics of self-incompatibility in *Brassica*. Annu. Rev. Genet. **23,** 121–139.

**O'Farrel, P.H.** (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250,** 4007–4021.

**Sommer, H., Beltrán, J.-P., Huijser, P., Pape, H., Lönnig, W.-E., Saedler, H., and Schwartz-Sommer, Z.** (1990). *Deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: The protein shows homology to transcription factors. EMBO J. **9,** 605–613.

**Timberlake, W.E.** (1980). Developmental gene regulation in *Aspergillus nidulans*. Dev. Biol. **78,** 497–510.

**Van Ness, J., and Hahn, W.E.** (1982). Physical parameters affecting the rate and completion of RNA driven hybridization of DNA: New measurements relevant to quantitation based on kinetics. Nucl. Acids Res. **10,** 8061–8077.

**Watson, C.J., and Jackson, J.F.** (1985). An alternative procedure for the synthesis of double-stranded cDNA for cloning in phage and plasmid vectors. In DNA Cloning: A Practical Approach, Vol. 1, D.M. Glover, ed (Oxford: IRL Press), pp. 79–88.

**Yanofsky, M.F., Ma, H., Bowmann, J.L., Drews, G.N., Feldmann, K.A., and Meyerowitz, E.M.** (1990). The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. Nature **346,** 35–39.

**Zimmermann, C.R., Orr, W.C., Leclerc, R.F., Barnard, E.C. and Timberlake, W.E.** (1980). Molecular cloning and selection of genes regulated in *Aspergillus* development. Cell **21,** 709–715.