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Identification of Genes Expressed in the Tobacco Shoot Apex during the Floral Transition

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The shoot apex of higher plants contains undifferentiated meristematic cells that serve as the origin of post-embryonic organs. The transition from vegetative to reproductive growth results in the commitment of the apical meristem to floral organ formation. To identify the molecular signals that initiate floral development, we have pursued the isolation of genes that are transcriptionally active in the shoot apex of tobacco during the transition from vegetative to floral growth. The small size of the apex led us to utilize polymerase chain reaction technology for the differential screening of an unamplified, subtracted cDNA library made from transition shoot apices. This approach enabled the isolation of the apex-specific and floral apex-specific cDNA clones described in this paper. One clone, A3, detected an equivalent level of transcript in the shoot apex during all developmental stages observed. The second clone, FA2, detected a unique transcript that increased in abundance in the shoot apex during the transition to flowering and showed high levels of expression in developing petals, stamens, and pistils.

INTRODUCTION

A critical developmental event in higher plants is the transition from vegetative to reproductive growth, which is marked by the onset of flowering. During the vegetative phase of plant development, the shoot apical meristem functions in an indeterminate fashion to repetitively generate leaves, stem, and quiescent axillary meristems while replenishing apical meristem cells (McDaniel, 1980; for reviews, see Wardlaw, 1965; Steeves and Sussex, 1989). In many angiosperms, the transition to flowering, induced by developmental age, photoperiod, or temperature, restricts the shoot apical meristem to floral development. Ultimately, this results in the consumption of the apical meristem by the production of determinate floral meristems and the subsequent development of reproductive organs (Hicks and Sussex, 1971; McDaniel et al., 1985; Larkin et al., 1990).

Physiological studies with several plant species indicate that the induction of flowering can be regulated by both promotive and inhibitory, graft-transmissible substances (Evans, 1960; Lang et al., 1977; for review, see Bernier, 1988). These as-yet-unidentified substances influence the cells of the shoot apex by controlling the process of floral evocation that begins the vegetative-to-floral transition (Evans, 1969). The floral transition encompasses the organization of the inflorescence and flower meristems and the initiation of floral organ primordia. Floral organ initiation occurs in a precise, determinate pattern, and even the destruction of some organ primordia early in development does not affect the initiation and development of the remaining floral organs (Hicks and Sussex, 1971).

Changes in RNA and protein synthesis occur in the cells of the shoot apex during the vegetative-to-floral transition, suggesting that changes in gene expression are involved in the establishment of floral meristems (for reviews, see Bernier et al., 1981; Bernier, 1988). Further evidence for the genetic control of floral development comes from the identification of mutants with altered floral morphogenesis. Flowering mutants of *Arabidopsis* have recently been characterized, and the genes identified by these mutations are being isolated (Koornneef et al., 1983; Komaki et al., 1988; Bowman et al., 1989; Kunst et al., 1989). Already one such gene has been cloned from *Antirrhinum* by making use of differential gene expression in wild-type and mutant flower buds (Sommer et al., 1990). However, many plant species for which there exists a wealth of information on floral induction and apex transition are not suitable for detailed genetic analysis by classical methods. One such species, tobacco, features both day-neutral and photoperiod-sensitive cultivars, several tissue culture systems useful for flowering studies (Tran Thanh Van, 1973; Singer and McDaniel, 1987), and efficient methods of genetic transformation and plant regeneration.

We have begun a molecular analysis of the early steps in flowering by identifying genes transcribed in cells of the shoot apex of tobacco during the floral transition. Examination of the molecular mechanisms that regulate the

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transcription of these genes will be an important first step toward the identification of processes involved in the change from vegetative to floral growth. Such genes have been elusive in molecular cloning experiments because of the difficulty in obtaining sufficient amounts of apex tissue for the construction and screening of apex cDNA libraries (Bernier, 1988; Drews and Goldberg, 1989). In this paper, we describe the isolation and transcriptional patterns of two cDNA clones that represent genes expressed in the shoot apex during the transition to flowering. These clones were isolated from a floral transition apex cDNA library that was differentially screened with polymerase chain reaction (PCR)-amplified cDNA probes. This procedure should be applicable to a variety of experimental systems in which as little as 1.0 μg of poly(A)$^+$ RNA is available for cDNA library construction, screening, and confirmation of recombinant clones.

RESULTS

Description of Vegetative, Transition, and Floral Shoot Apices

Recognizable morphological changes occur in the tobacco shoot apex during plant growth and serve as landmarks for the developmental state of the apical meristem (Waterkeyn et al., 1965; Thomas et al., 1975; Gebhardt and McDaniel, 1987). Figure 1 shows the changes that were

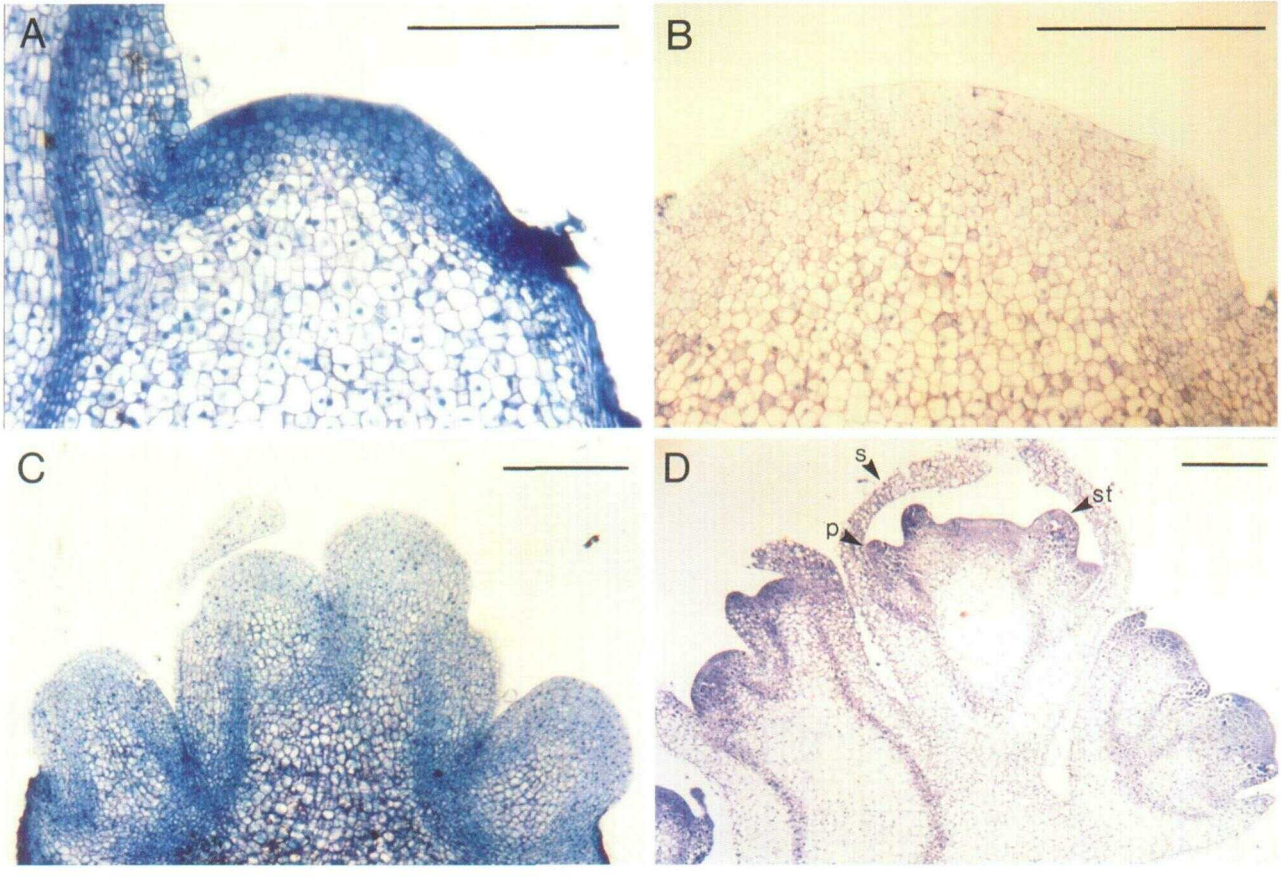

Figure 1. Photomicrographs of Sectioned Shoot Apices of *Nicotiana tabacum* cv Samsun at Different Stages of Development.

(A) Vegetative apex.
(B) Early transition apex showing initial doming.
(C) Late transition apex with multiple axillary inflorescence primordia and the terminal flower primordium.
(D) Floral apex characterized by the appearance of flower primordia, which show developing floral organs. Visible organ primordia of the terminal flower are labeled. s, sepal; p, petal; st, stamen.
Sections were prepared as described in Methods and stained with methylene blue/Azure II. Bars = 0.25 mm.

used as indicators of the developmental state of the shoot apex. Vegetative apices are conical and possess a small group of meristematic cells (Figure 1A). The first indication of the transition to flowering is the expansion and doming of the meristematic zone of the shoot apex. We refer to this as the early transition stage (Figure 1B). As the floral transition progresses, the terminal flower meristem and several axillary inflorescence meristems arise from the apical meristem (Kanchanapoom and Thomas, 1987), resulting in a late transition stage shoot apex (Figure 1C). Although no reproductive organ primordia are visible in transition apices, these apices and those of more developed plants are irreversibly committed to floral development, as demonstrated in photoperiod shift (Waterkeyn et al., 1965; Gebhardt and McDaniel, 1987) and rerooting experiments (Singer and McDaniel, 1986; Gebhardt and McDaniel, 1987; R.A. White and D.R. Meeks-Wagner, unpublished results). Unlike transition apices, floral apices (Figure 1D) possess reproductive organ primordia on the terminal flower bud.

Construction of the Transition Apex cDNA Library and PCR Amplification of Double-Stranded cDNAs

The appearance of a transition apex represents the earliest visible indication that the meristem is committed to floral development. Transition apices were chosen as the source of tissue for the construction of a cDNA library in an attempt to isolate genes that are transcribed during the early stages of flowering. Poly(A)$^+$ RNA was prepared from excised apices of greenhouse-grown tobacco, as described in Methods. An enrichment of apex-specific transcripts was achieved by the subtraction of messages common to transition apices and leaves of flowering plants. Double-stranded (ds) cDNA was subsequently synthesized and cloned into a phage λ cDNA vector, λCAT (C. Martin, M. Palazzolo, M. Strathmann, and E. Meyerowitz, unpublished results). Because of the small size of the shoot apical meristem, apex-derived mRNAs were difficult to

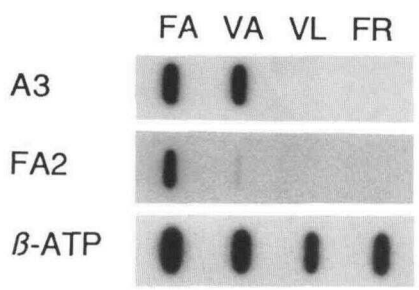

Figure 2. Secondary Screen of cDNA Clones FA2 and A3 from the Subtracted Transition Apex cDNA Library.

Two hundred nanograms of PCR-amplified double-stranded cDNA from floral apices (FA), vegetative apices (VA), leaves of vegetative plants (VL), and roots of flowering plants (FR) was transferred onto a nylon membrane. Each filter was hybridized with ^{32}P-labeled, PCR-generated DNA fragments of FA2 and A3, and with a ^{32}P-labeled tobacco ATPase β-subunit cDNA (β-ATP).

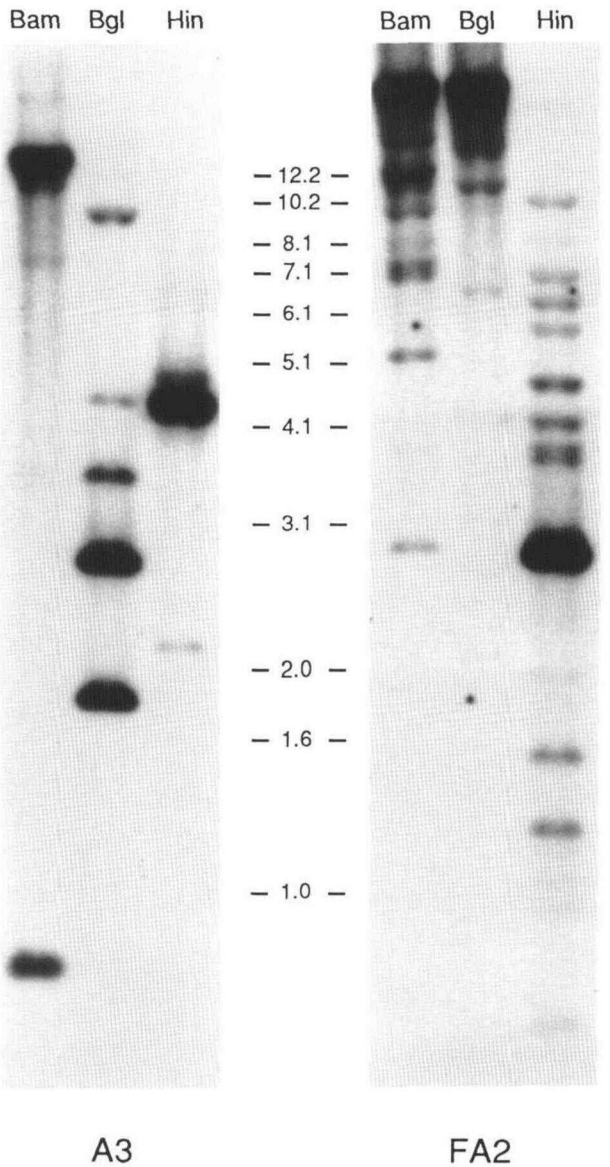

Figure 3. DNA Gel Blot Analysis of Tobacco DNA with A3 and FA2 Probes.

DNA isolated from leaves was digested with BamHI, BglII, or HindIII, electrophoresed through agarose gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled A3 or FA2 cDNA probes. Size marker units are in kilobases.

obtain in quantity. Thus, we utilized PCR amplification of cDNAs generated from floral apex and vegetative apex poly(A)$^+$ RNA to prepare sufficient quantities of ds-cDNA for preliminary screens of the transition apex cDNA library. This material was also used in secondary screens for the confirmation of putative tissue-specific clones.

Screening the Transition Apex cDNA Library

Two strategies were used for the primary screens of the transition apex cDNA library: (1) screening for clones of genes specifically expressed in the shoot apex at equivalent levels throughout development, and (2) screening for clones of genes specifically activated in the shoot apex during the transition to flowering.

The identification of apex-constitutive genes was accomplished by screening the transition apex cDNA library with ^{32}P-labeled, PCR-amplified ds-cDNAs derived from floral apices (FA), leaves of vegetative plants (VL), and roots of flowering plants (FR). Approximately 2500 plaques from the transition apex library were screened, and 14 putative apex-specific positives were found. As a secondary screen, the insert cDNA from each clone was labeled with ^{32}P and used to probe slot blots containing PCR-amplified ds-cDNA from FA, VL, FR, and vegetative apices (VA). Figure 2 shows the transcript patterns of a representative apex-specific clone, A3 (apex clone 3). cDNAs specific to clone A3 were observed in the FA and VA samples, but virtually undetectable in VL and FR ds-cDNA populations. An identical slot blot was hybridized with a ^{32}P-labeled tobacco β-ATPase cDNA fragment (Boutry and Chua, 1985). β-ATPase gene transcripts are found at nearly equivalent levels in all tobacco tissues examined (Kuhlemeier et al., 1987), and, thus, this cDNA was used as a probe to ensure equal amounts of poly(A)$^+$ RNA in each sample.

The isolation of transition apex-specific and floral apex-specific clones was accomplished by screening approximately 3000 plaques from the transition apex cDNA library with FA and VA ds-cDNA probes; 14 putative floral-specific differentials were found. These clones were also subjected to secondary screens to examine transcription patterns in FA, VA, VL, and FR samples. Figure 2 shows the second-

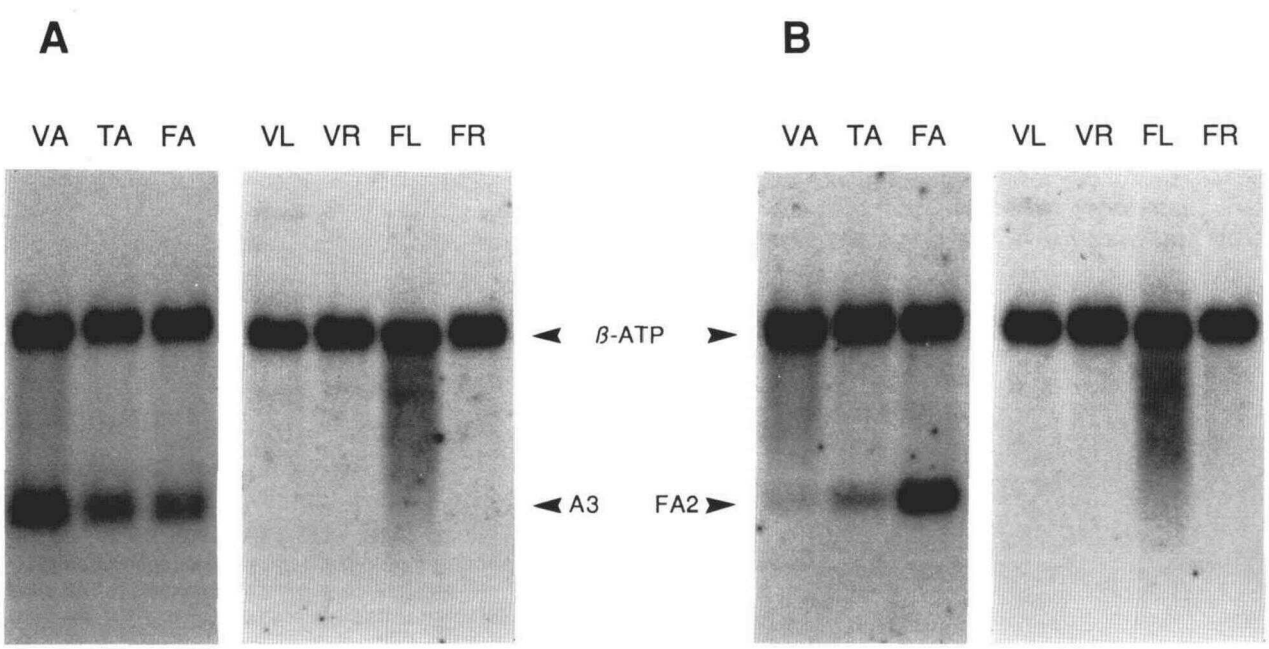

Figure 4. RNA Gel Blot Analysis of A3 and FA2 Transcripts. Poly(A)$^+$ RNA was isolated from vegetative apices (VA), transition apices (TA), floral apices (FA), leaves of vegetative plants (VL), roots of vegetative plants (VR), leaves of flowering plants (FL), and roots of flowering plants (FR). One hundred fifty nanograms of each RNA was electrophoresed through formaldehyde agarose gels and transferred onto nylon membranes.

(A) Sequential hybridization with ^{32}P-labeled probes generated from the A3 cDNA fragment and tobacco ATPase β-subunit cDNA (β-ATP).
(B) Sequential hybridization with ^{32}P-labeled probes generated from the FA2 cDNA fragment and tobacco ATPase β-subunit cDNA (β-ATP). As determined by scanning densitometry of several autoradiographs, signal intensities relative to those of the weakest apex signal are: A3: vegetative apex, 1.3×; transition apex, 1.1×; floral apex, 1.0×. FA2: vegetative apex, 1×; transition apex, 10×; floral apex, 45×.

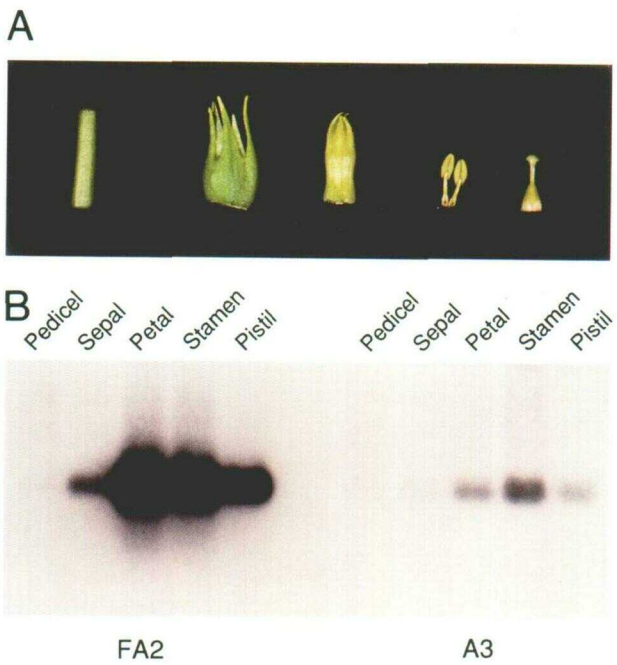

Figure 5. RNA Gel Blot Analysis of FA2 and A3 Transcripts in Developing Floral Organs.

(A) Organs from a stage-2 flower bud representative of those used for poly(A)$^+$ RNA isolation. Left to right: pedicel, sepals, petals, stamens, pistil.

(B) Two hundred nanograms of poly(A)$^+$ RNA from each sample was used for RNA gel blot analysis. ^{32}P-labeled A3 and FA2 fragments of equal specific activity were used as hybridization probes, and the filters were exposed for equivalent duration to autoradiographic film. The quantities of poly(A)$^+$ RNA used were standardized from results previously obtained with ^{32}P-labeled ATPase β-subunit (data not shown).

ary screen of one of these clones, FA2 (floral apex clone 2), which displayed a strong FA signal, a weak VA signal, and background signals from VL and FR samples. Because of their apex specificity, clones A3 and FA2 were chosen for further analysis.

Figure 3 displays autoradiographs of DNA gel blots of tobacco DNA that were probed with A3 and FA2 cDNA fragments. From the complex hybridization pattern, it appears that the gene represented by the FA2 cDNA fragment is a member of a multigene family; this was supported by the finding that an FA2-specific primer amplified more than one distinct band in PCR reactions utilizing tobacco cDNA as template (A. J. Kelly, unpublished results). The A3 probe hybridized to a small number of restriction fragments. This may indicate that few genes exist in the tobacco genome that share sequence similarity with the A3 cDNA fragment.

A3 and FA2 Transcripts Are Found in Shoot Apices but not in Leaves or Roots

The RNA gel blot analysis shown in Figure 4A demonstrated that A3 transcript levels varied no more than 30% among the three stages of apex development examined, as determined by scanning densitometry. Also, the 900-nucleotide A3 transcript was undetectable, even in long exposures of autoradiographs, in samples of poly(A)$^+$ RNA from leaves of vegetative and flowering plants, and from roots of vegetative and flowering plants (Figure 4A). RNA gel blot analysis of the FA2 transcript with poly(A)$^+$ RNA from floral apices, transition apices, and vegetative apices detected a distinct 900-nucleotide transcript that was 45 times more abundant in floral apices as compared with vegetative apices (Figure 4B). FA2 transcript could not be detected, even in very long exposures, in samples of leaf and root poly(A)$^+$ RNA (Figure 4B). Thus, the patterns of tissue specificity seen with the PCR-amplified cDNA accurately reflected those patterns seen with the corresponding poly(A)$^+$ RNA, substantiating the use of PCR material for screening the cDNA library.

Further analysis of the tissue specificity of A3 and FA2 transcripts was performed with poly(A)$^+$ RNA prepared from tissues harvested from plants of various ages. These tissues included root from nine age groups (spanning seedling to mature flowering plant), and leaf and stem from five positions on a flowering plant. The quantities of poly(A)$^+$ RNA used were identical to those described for Figure 4, and in no case was a hybridization signal detected when the poly(A)$^+$ RNAs were probed with ^{32}P-labeled A3 and FA2 cDNA fragments (data not shown). Based upon the relative exposure times of the blots, we estimated that A3 and FA2 transcripts must be at least 250 times more abundant in floral apices than in leaves and roots.

FA2 Transcript Is Abundant in Immature Petals, Stamens, and Pistils

Because the mRNA detected by clone FA2 was restricted to the shoot apex and increased as the apex progressed through the floral transition, we examined young floral organs for the presence of FA2 transcript. Poly(A)$^+$ RNA was isolated from floral organs excised from 1.0-cm to 2.0-cm buds (developmental stages 2 to 4; Goldberg, 1988); representative organs are shown in Figure 5A. RNA gel blot analysis with these samples demonstrated a dramatic increase in the FA2 transcript level in the inner whorls of floral organs (petals, stamens, and pistils) (Figure 5B). Approximately 20 times more FA2 transcript was detected in petals as compared with floral apices. A3 transcript was also most abundant in these inner whorls of organs, although not as abundant as FA2 transcript; A3 transcript

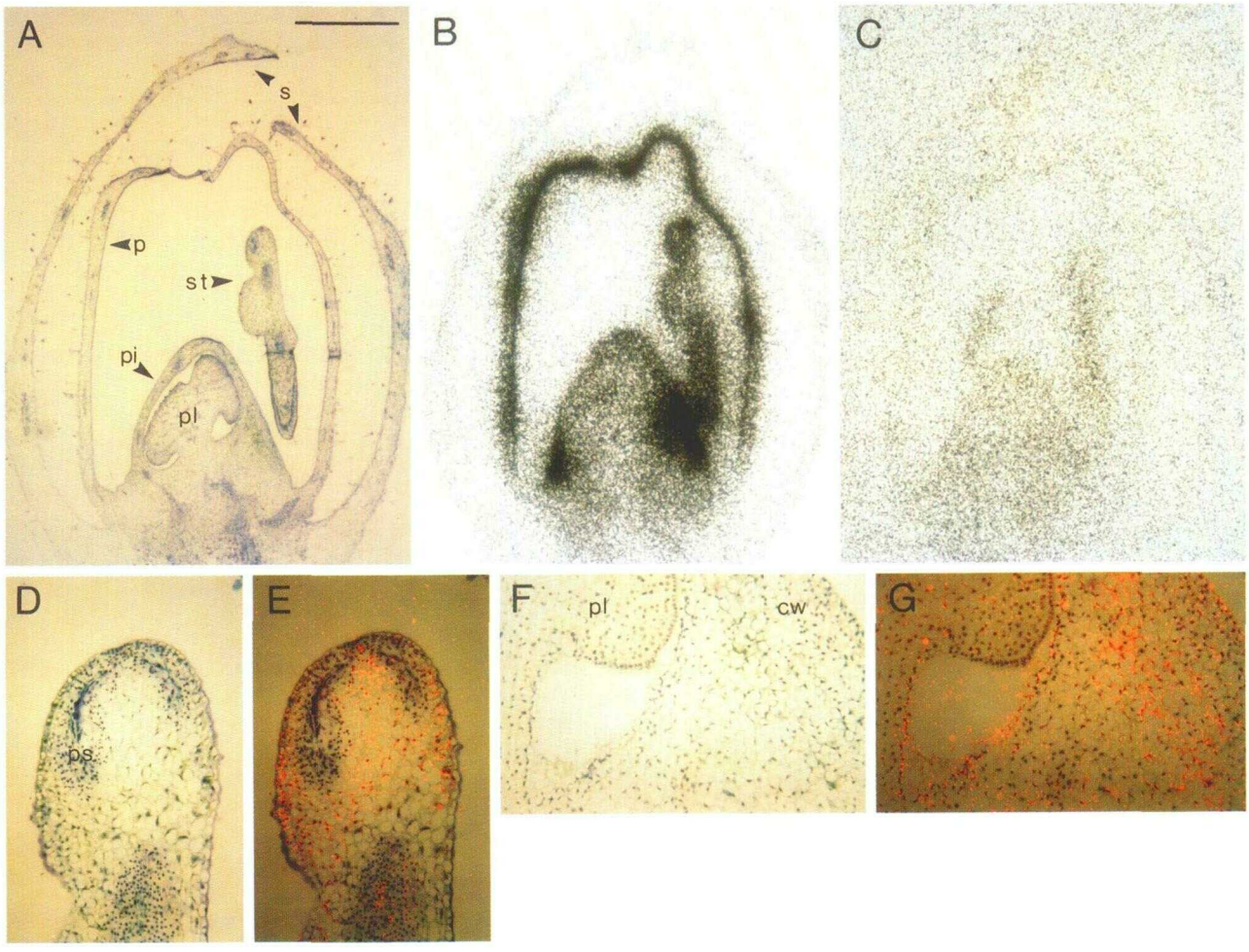

Figure 6. Localization of FA2 RNA in Cells of an Immature Flower Bud by in Situ Hybridization.

Longitudinal sections were made of a flower bud which was 5 mm in length [before stage "1" as defined by Goldberg (1988)]. Hybridization was performed with ^{32}P-labeled antisense or sense RNA probes made from the FA2 cDNA insert. Hybridized sections were exposed to single-emulsion film and, subsequently, to liquid photographic emulsion. The sections were then stained with methylene blue/Azure II. Double-exposure photographs in **(E)** and **(G)** were made by first photographing the section under bright-field illumination and then photographing the same section under dark-field illumination with a red (Wratten No. 25A) filter in the illumination source.

(A) Bright-field photograph of a longitudinal section of a flower bud.

(B) Bright-field photograph of the single-emulsion film after exposure to the section pictured in **(A)**, which was hybridized with FA2 antisense RNA probe.

(C) Bright-field photograph of the single-emulsion film after exposure to a section of the flower bud hybridized with FA2 sense probe. This is a fivefold overexposure relative to the exposure shown in **(B)**.

(D) Bright-field photograph of the terminal portion of the stamen showing the developing pollen sac.

(E) Double-exposure photograph of the same region of the stamen as in **(D)** showing the hybridization of FA2 antisense RNA probe. Silver grains in the liquid emulsion appear as orange dots over the cells.

(F) Bright-field photograph of the base of the pistil showing the carpel wall and ovary.

(G) Double-exposure photograph of the same region of the pistil as in **(F)** showing the hybridization of FA2 antisense RNA probe. s, sepal; p, petal; st, stamen; pi, pistil; ps, pollen sac; cw, carpel wall; pl, placenta. Bar = 1 mm.

levels in the stamen sample were equivalent to the amount of A3 RNA in the floral apices.

In Situ Localization of FA2 Transcript in Immature Floral Organs

Detection of FA2 transcript in perianth organs, as well as in reproductive organs, suggested that the gene represented by FA2 is not directly involved in gametogenesis and that FA2 transcripts would not occur in cell types intimately related to this process. The localization of FA2 RNA to cells of an immature flower bud is shown in Figure 6. In situ hybridizations confirmed the pattern of FA2 transcripts observed in young floral organs by RNA gel blot analysis (see Figure 5): high levels of FA2 transcript were found in petals, stamens, and pistils, but not in sepals (Figure 6B). Examination of hybridization patterns showed that FA2 RNA occurs in the parenchyma cells of the anther and filament, but not in the cells of the developing pollen sac (Figures 6B and 6E). FA2 RNA was detected in parenchyma cells of the carpel wall, predominantly at the base (Figures 6B and 6G), and in the internal epidermal cells of the carpel wall (Figure 6G). Thus, FA2 RNA was not associated at this stage with cells of the pollen sac or placenta, structures directly involved in the formation of pollen or ovules, respectively.

DISCUSSION

Beginning with very small quantities of poly(A)$^+$ RNA, we have isolated several cDNA clones representing gene families transcriptionally expressed in the tobacco shoot apex during the transition to flowering. The use of PCR-amplified ds-cDNA populations has allowed us to circumvent the constraints imposed by the small size and time-consuming isolation of apex tissue. From 1.5 ng of single-stranded cDNA, we have routinely produced 10 μg to 15 μg of a PCR-amplified ds-cDNA. Using the procedure described in this paper, we estimate that less than 1 μg of poly(A)$^+$ RNA can provide enough cDNA to construct an unamplified, subtracted cDNA library and to execute both primary and secondary screens of such a library.

Construction of a cDNA library from PCR-amplified ds-cDNA (Belyavsky et al., 1989; Timblin et al., 1990) would further reduce the quantity of apex poly(A)$^+$ RNA required for isolating apex-specific cDNA clones. This strategy was not pursued in the construction of our transition apex cDNA library to ensure that potential PCR artifacts were not permanent features of the library. To further minimize possible artifacts created by PCR amplification, the primary and secondary screens were comparisons of PCR-amplified ds-cDNA populations prepared under equivalent amplification parameters. All of the cDNA clones we have examined displayed identical tissue-specific transcript levels when assayed by slot blots containing PCR-amplified cDNA and by RNA gel blot analysis with the corresponding poly(A)$^+$ RNA samples.

Clones A3 and FA2 represent two of the apex-specific cDNAs that we have identified by screening the transition apex cDNA library. Transcripts homologous to A3 or FA2 were not detected in any leaf, root, or stem tissues examined (Figure 4). A3 transcript occurred at a nearly constant level in the shoot apex throughout the transition to flowering (Figure 4A), and it did not increase in abundance in developing floral organs (Figure 5). FA2 transcript, however, became more prevalent in the shoot apex as the plant initiated flowering (Figure 4B). Very small amounts of FA2 transcript were detected in mature vegetative apices. This may represent a low level of transcription before the commitment of flowering, or it may reflect contamination of the vegetative apex sample with transition apices. In comparison with the amount of FA2 transcript in mature vegetative apices, the levels increased approximately 10-fold in transition apices and 45-fold in floral apices. Unlike A3 mRNA, the level of FA2 transcript increased dramatically in developing floral organs, particularly in the petals, stamens, and pistil (Figure 5). The occurrence of FA2 transcript has also been investigated in tobacco thin cell layer (TCL) explants, which produce either vegetative shoots or flower buds in tissue culture (Tran Thanh Van, 1973; Meeks-Wagner et al., 1989). FA2 transcripts were detected only in floral TCL explants, and the accumulation of FA2 mRNA correlated with the appearance of flower buds (G.K. Reid and D.R. Meeks-Wagner, unpublished results).

At this stage of our analysis, it is not possible to reliably predict the functions of the A3 and FA2 gene products. A3 transcripts were observed at constant levels in the shoot apex throughout the floral transition, and, thus, the gene represented by this cDNA may encode functions involved in the production of cell types found in both vegetative and floral organs. For example, the procambial cells of the meristem initiate vascular tissue, which is found in the stem and leaf, as well as in the sepal, petal, stamen, and pistil. In contrast, the FA2 cDNA fragment detected increasing levels of transcript in the shoot apex during the transition to flowering. Thus, the FA2 gene product is likely to be involved in a floral-specific process. The observation that high levels of FA2 transcript occurred in the inner whorls of floral organs of immature flower buds (Figure 5) and that FA2 mRNA is not detected in cells directly associated with gametogenesis (Figure 6) suggests that this gene family is expressed in the meristematic cells involved in a more general aspect of floral organogenesis.

Previous studies predict that gene expression in the shoot apex during the transition to flowering is a critical regulatory aspect of the flowering process. Wardlaw (1957) suggested that the meristem is a "reaction center" in which genetic determinants control the sequential de-

velopment of floral organs. When applied to the shoot apex during the floral transition, inhibitors of RNA synthesis disrupt the flowering response of several photoperiodic species (Evans, 1964; Kinet et al., 1971). The detection of FA2 transcript in the transition apex confirms that genes expressed in developing floral organs can be active very early during the flowering process, that is, before the appearance of floral organ primordia.

Photoperiod-sensitive plants have been widely used for investigations of floral induction and evocation. A single gene mutation in a day-neutral variety of tobacco has generated the short-day responsive cultivar Maryland Mammoth (Allard, 1919). We recently have found that FA2 transcript increased in shoot apices of Maryland Mammoth plants that had been shifted to inductive short days (L.A. Tolar and D.R. Meeks-Wagner, unpublished results). The use of such cultivars will, thus, be extremely valuable in our investigations into the transcriptional regulation of the gene represented by the FA2 cDNA fragment. A comparison of A3 and FA2 regulatory sequences may reveal *cis*-acting elements responsible for the apex-specific accumulation of transcripts and for the increased levels of FA2 transcript seen during the floral transition.

METHODS

Plant Material and Apex Collection

Nicotiana tabacum cv Samsun, a day-neutral cultivar of tobacco, was grown in soil in a greenhouse. Before apex excision, plants were estimated to be vegetative, transition, or floral, based upon the number of leaves of 3 cm or greater in length. Generally, vegetative apices were collected from plants with six to eight leaves >3 cm in length, transition apices were collected from plants with 10 to 13 leaves, and floral apices were harvested from plants with 15 to 18 leaves. The uppermost leaves were then removed and the developmental stage was confirmed as vegetative, transition, or floral, based upon apex morphology. Apices were regarded as vegetative if no doming of the meristem was obvious (Figure 1A). Transition apices ranged from those with expanded, domed meristems (Figure 1B) to apices having several inflorescence meristems; sepal primordia, but no other developing floral structures, were sometimes evident on the terminal flower and axillary inflorescence primordia (Figure 1C). Apices displaying petal, stamen, and carpel primordia on one or more buds were classified as floral (Figure 1D). A correlation between apex morphology and commitment to floral development had been previously determined under our growth conditions by rerooting experiments similar to those described by Singer and McDaniel (1986) (R.A. White and D.R. Meeks-Wagner, unpublished results). During excision, care was taken to remove as much stem and leaf primordia tissue as possible. Apices were immediately placed on dry ice and stored at −80°C until thawed for RNA extraction.

Tissue Sectioning and In Situ Hybridizations

Apices were excised from greenhouse-grown plants, fixed in glutaraldehyde, and dehydrated in a graded ethanol series. Vegetative apices and early and late transition apices shown in Figure 1 were embedded in LR Gold resin (Polysciences Inc., Warrington, PA) as described by McFadden et al. (1988); floral apices were embedded in a similar fashion in diethylene glycol distearate-paraffin (DGD) (Polysciences Inc.). Sections of 3-μm to 4-μm thickness were made with glass knives in an ultramicrotome. Sections were fixed onto glass microscope slides using polyvinyl alcohol-vinyltriethoxysilane adhesive, stained with methylene blue/Azure II, and photographed under bright-field illumination.

Localization of RNA was performed with ^{32}P-labeled, in vitro synthesized RNA probes generated from T7 and SP6 promoters on the λCAT-derived plasmid containing the FA2 cDNA fragment (see below). DGD-embedded sections of flower buds were mounted onto glass slides with polyvinyl alcohol-vinyltriethoxysilane adhesive. The prehybridization, hybridization, and wash conditions were essentially the same as those described by Cox and Goldberg (1988). Hybridizations were performed for 16 hr to 18 hr at 42°C with approximately 5×10^4 cpm/μL probe. After the final wash, the slides were briefly rinsed in a graded ethanol series, air dried, and placed on SB-5 single-side emulsion film (Kodak). Selected slides were coated with NTB-2 photographic emulsion (Kodak) and exposed at 4°C; the slides were developed in D-19 developer (Kodak) for 4 min at 15°C, fixed, washed, and stained with methylene blue/Azure II.

RNA Isolation

Total RNA was extracted from tissues as described by Mohnen et al. (1985). Apex tissue was treated in a similar fashion except that tissue homogenization was achieved by manual grinding of the apices with glass pestles in 1.5-mL microcentrifuge tubes in the presence of Tris-saturated phenol. Poly(A)$^+$ RNA was isolated by passage over oligo(dT)-cellulose (Maniatis et al., 1982).

Construction of the Subtracted Transition Apex cDNA Library

Two micrograms of poly(A)$^+$-enriched RNA was isolated from 97 mg of transition tobacco apex tissue and converted into radiolabeled first-strand cDNA by avian myeloblastosis virus reverse transcriptase using primer No. 525 [5′-CCCGGGAAGCTTA-AGC$(T)_{14}$] essentially as described by Huynh et al. (1985). The transition apex cDNA was subtracted twice with poly(A)$^+$ RNA from leaves of flowering plants using the biotinylation/phenol extraction procedure described by Duguid et al. (1988) (Invitrogen Corp., San Diego, CA). After the second subtraction, 5% of the transition apex cDNA remained (approximately 4 ng) and was converted into ds-cDNA with DNA polymerase, treated with S1 nuclease, methylated with EcoRI methylase, and repaired using both the Klenow fragment of DNA polymerase I and T4 DNA polymerase. This transition apex cDNA was then ligated with kinased EcoRI linkers (5′-GGAATTCC-3′) and digested with HindIII and EcoRI restriction enzymes to facilitate directional cloning into a bacteriophage λ cDNA cloning vector. After the digestion, products were fractionated on a 25-cm × 2-mm Bio-Gel A-5M gel filtration column (Bio-Rad Laboratories), and the peak fractions were ligated with phage arms derived from an EcoRI/HindIII digestion of λCAT (kindly provided by E. Meyerowitz). This vector allows the directional cloning of cDNA fragments, strand-specific synthesis of cRNA using either the T7 or SP6 promoter, and the generation of plasmid molecules containing the cDNA

fragment by recombination of the two defective ampicillin genes when the recombinant phage are grown in a *recA*$^+$ *Escherichia coli* host. The ligation mix was packaged using Gigapack II Gold packaging extract (Stratagene) and titered on *E. coli* MC1061 (*recA*$^-$). This procedure produced a subtracted transition apex cDNA library of 2×10^7 recombinant phage.

PCR Amplification of ds-cDNA

PCR amplification of cDNA molecules was performed by a modification of the RACE procedure of Frohman et al. (1988). Poly(A)$^+$ RNA isolated from FA, VA, VL, and FR was first converted into single-stranded cDNA as described above. The 3′ ends of these molecules were extended by the addition of approximately 20 to 30 deoxyadenosine (dA) nucleotides using dATP and terminal transferase. Each dA-tailed cDNA was then set up for PCR amplification using the following conditions: 1.5 ng of dA-tailed cDNA; 67 mM Tris-HCl, pH 8.8; 6.7 mM $MgCl_2$; 2 mM each dATP, dCTP, dGTP, and dTTP; 33 μg/mL primer No. 525; and 10% (v/v) DMSO in a total volume of 50 μL. After the reaction mixture was heated at 95°C for 5 min, 2.5 units of Taq polymerase was added and 35 μL of mineral oil was layered onto the solution. Amplification was performed in a Coy Thermocycler (Coy Laboratory Products, Inc., Ann Arbor, MI) programmed for 1 cycle of 60°C × 40 min; followed by 40 cycles of 95°C × 1 min, 47°C × 2 min, 60°C × 0.5 min, 71°C × 3 min; and a final extension at 71°C × 15 min. After PCR amplification, the ds-cDNA was extracted with chloroform and purified on Sephadex G-50 (Pharmacia LKB Biotechnology Inc.) spin columns as described by Maniatis et al. (1982). Typically, 10 μg to 15 μg of PCR-amplified ds-cDNA was produced from 1.5 ng of dA-tailed, single-stranded cDNA. Library probes were generated by random hexamer labeling (U.S. Biochemical Corp.) 0.5 μg of the appropriate PCR-amplified ds-cDNA with 150 μCi of α-^{32}P-dCTP.

Isolation of Cloned cDNA Inserts and Preparation of Probes from Inserts

The cDNA fragments from clones of interest were PCR-amplified using the procedure of Saiki et al. (1988) with primers complementary to the flanking λCAT DNA sequences. Before radiolabeling, the PCR-amplified cDNA fragments were purified on Sephadex G-50 spin columns, followed by ethanol precipitation. Alternatively, a plasmid containing the cDNA fragment was generated by in vivo recombination in *E. coli* MP23 (*recA*$^+$) infected with the corresponding phage and selection for ampicillin-resistant, plasmid-bearing recombinants. The cloned fragment was then gel purified from an EcoRI/HindIII digestion of the plasmid and radiolabeled with α^{32}P-dCTP by random hexamer priming (U.S. Biochemical Corp.).

Secondary screens of selected clones were performed by blotting 200 ng of each PCR-amplified ds-cDNA (FA, VA, VL, and FR) through a slotted manifold onto a nylon membrane (Hybond-N; Amersham Corp.) These membranes were hybridized with ^{32}P-labeled DNA fragments generated from clones that showed differentials on the primary library screens, and with a ^{32}P-labeled fragment of the tobacco nuclear-encoded β-ATPase cDNA clone (Boutry and Chua, 1985; kindly provided by S. Kay and N.-H. Chua).

RNA and DNA Gel Blot Analyses

For RNA gel blot analysis, RNA was electrophoresed in 1% agarose, 6.8% formaldehyde gels and blotted onto nylon membranes as described by Maniatis et al. (1982). Random hexamer-primed labeling was used to generate hybridization probes from plasmid-derived, agarose gel-purified cDNA fragments of A3, FA2, and β-ATPase. Hybridizations were carried out in aqueous buffer at 60°C to 65°C according to the manufacturer's instructions (Amersham) for approximately 40 hr. Blots were washed three times for 15 min each at 65°C in 2 × SSC, 0.1% SDS followed by three 15-min washes at 65°C in 0.2 × SSC, 0.1% SDS. Autoradiographic exposures were carried out at −80°C with intensifying screens on Kodak X-OMAT AR film. DNA gel blot analysis was performed by digestion of 5 μg of tobacco genomic DNA with restriction enzymes, followed by electrophoresis in 0.6% agarose gels. The gels were subsequently denatured and neutralized by standard procedures. Blots, hybridizations, washes, and autoradiographic exposures were carried out as described for RNA gel blot analysis.

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