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Tobacco Genes Expressed during in Vitro Floral Initiation and Their Expression during Normal Plant Development

D. Ry Meeks-Wagner,[a,1] Elizabeth S. Dennis,[a] Kiem Tran Thanh Van,[b] and W. James Peacock[a]

[a] Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, G.P.O. Box 1600, Canberra, ACT, 2601, Australia

[b] Institut de Physiologie Végétale, Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France

Since the transition from vegetative to floral development in plants is likely to be influenced by gene expression in several plant organs, we have used an in vitro system, the tobacco "thin cell layer" system as a model for investigating gene expression associated with the initiation of flowering in higher plants. cDNA cloning has been used to identify mRNAs abundant during thin cell layer floral initiation. These genes are expressed in thin cell layer explants initiating floral meristems but not in thin cell layer explants initiating vegetative shoot meristems or possessing roots. Two of these genes are expressed transcriptionally in incipient floral apices during normal plant development. Transcripts of these genes, plus a third gene, occur at low levels in several plant organs and at high levels in the roots, with the maximum levels of root expression reached just prior to the formation of floral meristems.

INTRODUCTION

In plants the transition to flowering marks the beginning of reproductive development during which meristems produce flowers rather than leaves, stem, and associated vegetative structures. Internal signals related to developmental age or external signals such as daylength or temperature can induce the onset of flowering, and there is evidence that both promotive and inhibitory substances are involved in the inductive process (Evans, 1960; Lang et al., 1977; reviewed by Bernier, 1988). It is well established that some of these substances are generated in leaf tissues (reviewed by Lang, 1965, and by Zeevaart, 1976), but other plant organs have also been observed to influence the induction, initiation, and development of flowers (see Bernier, 1986).

In normal circumstances floral induction results in the initiation (or evocation) of floral meristems and the commitment to flowering (Hicks and Sussex, 1971; McDaniel et al., 1985). The generation of floral meristems involves changes in cellular metabolism, cell elongation, and the rate and orientation of cell division. These changes lead not only to the generation of the germline and the organs for sexual reproduction but also to particular spatial relationships of these organs that often differ from the spacing characteristic of vegetative organs. The production of new gene products during floral initiation has been demonstrated (Pierard et al., 1980); however, their role in the formation of floral meristems has not been determined.

Although anatomical differences between vegetative and floral organs are substantial, several types of evidence suggest that leaves and floral organs are highly homologous. Morphological and evolutionary comparisons of the vegetative and floral structures of some plant groups have shown that floral organs are likely to be modified vegetative structures (Arber, 1937; Wilson and Just, 1939; Bailey and Swamy, 1951). In a number of plant species, heritable changes have been identified that result in the replacement of a floral organ, such as a petal, with a leaf-like structure (Meyer, 1966). Homology between floral and vegetative structures has also been demonstrated by nucleic acid hybridization analysis showing that a majority of mRNA species of tobacco floral structures (the anthers and ovaries) are common to the mRNA of tobacco leaves, whereas the mRNA populations of petals and leaves are at least 98% homologous (Kamalay and Goldberg, 1980).

Given the evidence for the similarity of overall patterns of gene expression between vegetative and floral organs, we reasoned that genes involved in the transition to flowering could be isolated by cDNA cloning and differential screening between mRNA populations of highly homologous vegetative and floral tissues at equivalent developmental stages. Because a number of plant organs may participate in steps important in the vegetative to floral transition, we avoided limiting our study to a particular plant organ or developmental time by using the tobacco

[1] To whom correspondence should be addressed. Current address: Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.

thin cell layer (TCL) tissue culture system (Tran Thanh Van, 1973) to produce experimental material. TCL explants derived from the inflorescence of flowering plants can directly form normal floral meristems and produce fertile flowers. Floral TCL explants must, therefore, express genetic information necessary for the initiation, formation, and development of floral meristems. In addition, by the manipulation of the culture conditions, TCL explants can be directed to form vegetative shoots or roots (Tran Thanh Van et al., 1974). In this paper we describe the isolation and characterization of genes expressed at an early stage of TCL floral initiation by the comparison of floral and vegetative explants. We also report on the spatial and temporal patterns of transcription displayed by some of these genes during normal plant development.

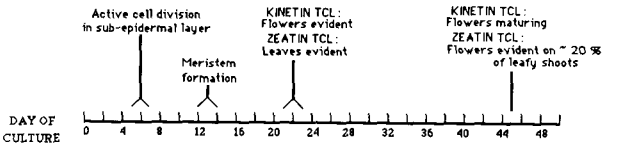

Figure 1. Sequence of Events during TCL Organogenesis.

Temporal order of recognizable "landmark" events during TCL floral bud (kinetin) and vegetative shoot (zeatin) organogenesis. This summarizes observations made by light microscopic examination of sections of TCL explants prepared as described for the in situ hybridization experiments (see "Methods").

RESULTS

Experimental System

Thin cell layers are small tissue explants approximately five cells thick consisting of fully differentiated epidermal, subepidermal, and parenchymal cells. Explants stripped from day-neutral tobacco *Nicotiana tabacum* cv. samsun initiate organogenesis from the subepidermal cells when floated on liquid tissue culture medium (Cousson and Tran Thanh Van, 1981; Tran Thanh Van et al., 1985). Depending on the composition of the culture medium, TCL explants taken from tobacco floral branches can be induced to form roots, leafy vegetative shoots (VS), or floral buds (FB) without intermediate callus formation (Tran Thanh Van et al., 1974; Tran Thanh Van et al., 1985); explants taken from the basal and intermediate zone of the plant have a reduced ability to form floral buds directly (Aghion-Prat, 1965; Tran Thanh Van et al., 1974). By using culture conditions described previously (Tran Thanh Van et al., 1985), the switch from VS to FB development was controlled solely by the type of cytokinin used in the medium. Zeatin resulted in vegetative shoots, whereas kinetin stimulated the direct formation of flowers and floral branches without any leaf production. These two cytokinins were the only variable component of the TCL cultures.

Under the culture conditions used, each explant produced fully differentiated organs according to the temporal scheme outlined in Figure 1. Examples of explants after 25 days of culture are shown in Figure 2. Vegetative shoots generated on zeatin medium will form flowers after producing at least three or four leaves; about 20% of the vegetative shoots produce floral buds after 45 days of culture. Although the temporal sequences of FB and VS organogenesis are comparable, there are obvious morphological differences between FB and VS explants. Floral development occurs in a polar fashion such that flower bud formation is almost exclusively localized to the basipetal end of the explant, whereas vegetative shoot formation normally occurs along the full length of the TCL explant; VS explants exhibit some additional callus growth, which is rare in FB explants.

Isolation of TCL Floral-Specific cDNA Clones

Day 7 of TCL development was chosen for cDNA library construction because in both FB and VS explants it was a stage of active cell division preceding meristem organization and organ differentiation. A cDNA library (Huynh et al., 1985) was made using poly(A^+) RNA from day 7 floral (FB7) TCL explants, and approximately 12,000 clones from the unamplified library were screened with ^{32}P-labeled cDNA from FB7 and day 7 vegetative shoot (VS7) explants. This primary screen identified 174 clones with enriched expression in day 7 floral bud explants. Additional screens verified 52 cDNA clones representing transcripts abundant in the mRNA population of FB7 explants and rare in the mRNA population of VS7 explants.

DNA hybridization homology between the clones classified the 52 cDNA clones into six gene families: the FB7 genes FB7-1 to FB7-6. Four families (FB7-1 to FB7-4) each contained more than 10 independently derived clones, whereas family FB7-5 contained two clones and FB7-6 was represented by a single cDNA clone. A clone designated cKZ1 was isolated for use as a control because it was expressed equally in both FB7 and VS7 explants. cKZ1 was shown subsequently to be homologous to the 28S ribosomal RNA of tobacco (data not shown), and probably resulted from rRNA secondary structure priming of first-strand cDNA synthesis.

FB7 Gene Expression Is Correlated with TCL Floral Initiation

To determine the temporal pattern of FB7 gene expression, we tested for the presence of FB7-1 to FB7-6 transcripts in the mRNA populations of FB explant cells prior

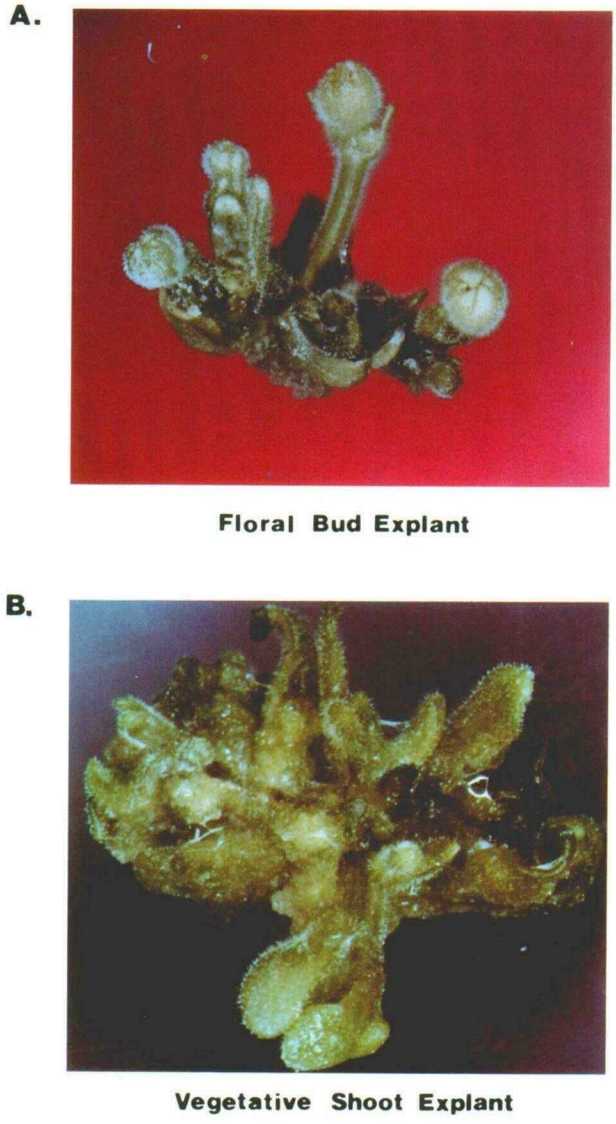

Figure 2. Floral and Vegetative Shoot TCL Explants.

Explants after 25 days of culture on medium containing either **(A)** kinetin (floral bud) or **(B)** zeatin (vegetative shoot) as the cytokinin. Photographs were taken with a macro lens; total magnification is approximately × 10.

to the initiation of culture and after 20 days of culture (FB20) when floral organs had differentiated. As shown in Figure 3, none of the FB7 genes showed significant expression in TCL explants prior to culture (Figure 3c); expression of all FB7 genes was detectable in FB20 explants (Figure 3d), but for all six families the level of expression was decreased relative to that found in FB7 explants (Figure 3, a and d).

These results demonstrate that the expression of FB7 genes is induced in floral TCL explants, but it is possible that this induction was caused by the presence of kinetin in the floral culture medium rather than by the initiation of floral development per se. This possibility was tested by examining FB7 gene expression in TCL explants derived from a lower region of the tobacco stem. Instead of floral development, vegetative shoot formation occurs when TCL explants from the lower stem are cultured on kinetin floral medium (Tran Thanh Van et al., 1974; K. Tran Thanh Van and D. R. Meeks-Wagner, unpublished results). Except for FB7-3, none of the FB7 genes displayed significant levels of transcripts in day 7 lower stem TCL explants cultured on kinetin medium (Figure 3b). We conclude that FB7-1, FB7-2, FB7-4, FB7-5, and FB7-6 are genes activated in association with floral initiation, whereas FB7-3 may represent a gene induced in kinetin TCL medium but not in zeatin TCL medium.

Molecular Characterization of the FB7-1, FB7-2, and FB7-5 Genes

DNA gel blot analyses (Southern, 1975) using *N. tabacum* cv. samsun DNA digested with several restriction endo-

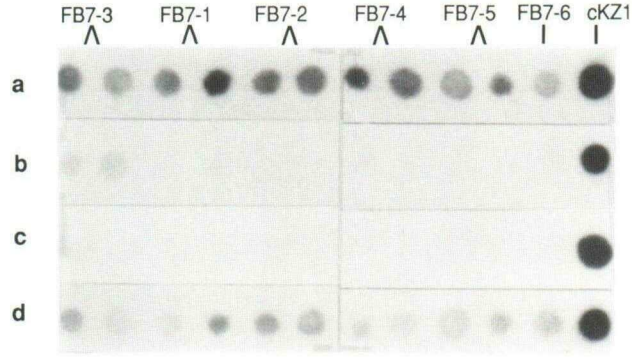

Figure 3. Hybridization of FB7 Sequences with cDNA Probes of Total poly(A^+) RNA from TCL Explants.

Recombinant λgt10 phage were spotted onto a lawn of restrictive *E. coli* (RY1075), allowed to form large plaques, and then transferred to nitrocellulose filters. High specific activity ^{32}P-labeled cDNA probes were made from poly(A^+) RNA by reverse transcriptase; equal amounts (cpm) of each probe were hybridized to the nitrocellulose filters. The cDNA probes were made from poly(A^+) RNA from **(a)** floral branch TCL explants at day 7 of culture on kinetin (floral medium, **(b)** intermediate zone TCL explants taken from the lower stem of flowering plants at day 7 of culture on kinetin (floral) medium, **(c)** floral branch TCL explants prior to culture (i.e., day 0), and **(d)** floral branch TCL explants at day 20 of culture on kinetin (floral) medium. cKZ1 (homologous to the 28S ribosomal RNA) was used as a positive control for hybridization.

nucleases showed that FB7-1, FB7-2, and FB7-5 are single or low copy number genes in tobacco (data not shown). Since these are not large gene families, and yet FB7-1 and FB7-2 were both isolated as more than 10 independent cDNA clones, then these genes must be frequent in the mRNA population of FB7 explants; FB7-5 may have slightly lower transcriptional activity in these explants.

RNA gel blot analysis (Thomas, 1980) was used to examine the transcriptional organization of FB7-1, FB7-2, and FB7-5. Single-strand RNA probes (Melton et al., 1984) were made from each DNA strand of the cDNA inserts of these genes and were hybridized to RNA gel blots containing poly(A^+) RNA from FB7 explants and VS7 explants as well as total RNA prepared from internode tissue of vegetative plants. Figure 4 shows that FB7-1, FB7-2, and FB7-5 each displayed homology to unique transcripts that were significantly more abundant in poly(A^+) RNA from FB7 explants than in poly(A^+) RNA from VS7 explants. In addition there was little hybridization of the FB7-1, FB7-2, and FB7-5 probes to sequences in the total RNA sample, and probes from only one DNA strand hybridized to poly(A^+) RNA, indicating that only one DNA strand is transcribed.

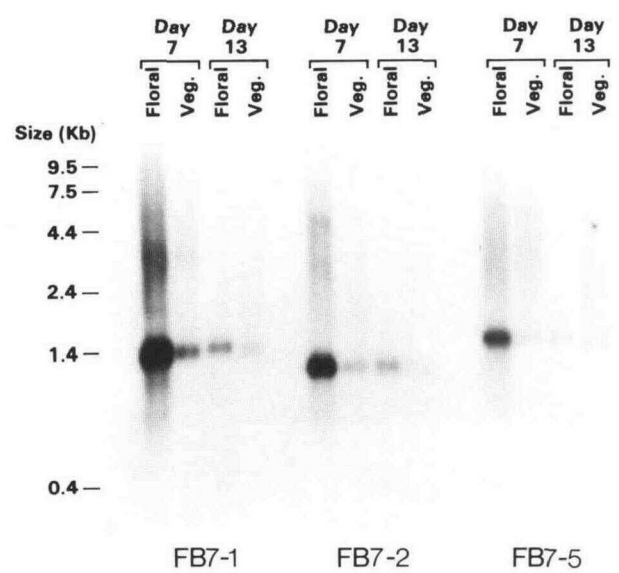

Figure 4. RNA Gel Blot Analysis of FB7 Expression during TCL Development.

Poly(A^+) RNA was isolated from day 7 and day 13 floral bud and vegetative shoot TCL explants. Equal amounts of RNA (1.0 μg) were fractionated by electrophoresis on a 1% formaldehyde agarose denaturing gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled single-strand RNA probes made from FB7-1, FB7-2, and FB7-5. An RNA ladder (Bethesda Research Laboratories) provided molecular size standards. Kb, kilobase.

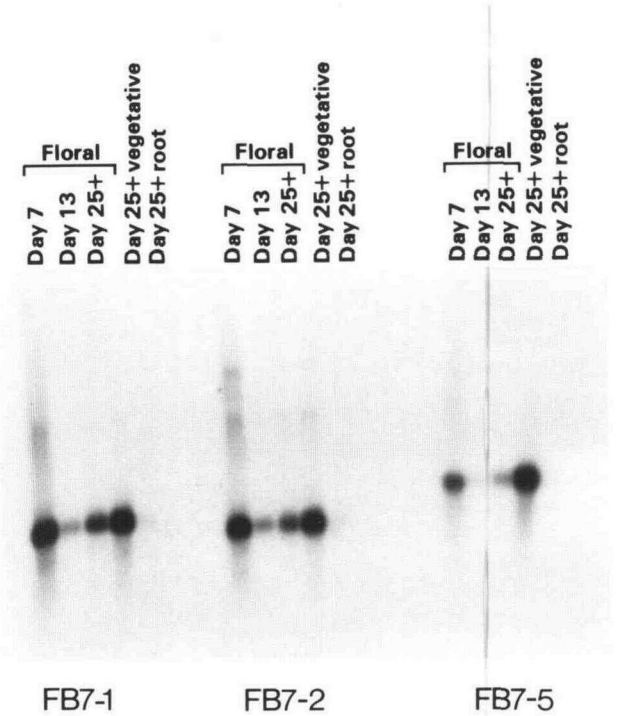

Figure 5. RNA Gel Blot Analysis of FB7 Expression during TCL Development.

Poly(A^+) RNA was isolated from day 7 and day 13 floral bud explants, and from day 25 to 33 combined (day 25+) of floral bud, vegetative shoot, and root TCL explants. Equal amounts of RNA (1.0 μg) were fractionated by electrophoresis on a 1% formaldehyde agarose denaturing gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled single-strand RNA probes made from FB7-1, FB7-2, and FB7-5.

Patterns of FB7-1, FB7-2, and FB7-5 Gene Expression in TCL Explants

A previous experiment (see Figure 3) suggested that FB7 gene expression was low in FB20 explants relative to the levels occurring in FB7 explants. RNA gel blot hybridization was used to define further the temporal pattern of FB7-1, FB7-2, and FB7-5 transcription in TCL explants during floral TCL culture, as well as during vegetative shoot and root culture. Three stages of TCL development were examined: (1) premeristem formation (day 7), (2) meristem formation (day 13), and (3) an interval of bud growth and organ differentiation (days 25 through 33, combined).

Figures 4 and 5 show that FB7-1, FB7-2, and FB7-5 displayed similar patterns of transcription in all situations examined. During FB explant culture, high levels of transcripts occurred at day 7, were reduced at day 13, but increased again at days 25 through 33. In VS explants the FB7 transcripts were at low levels at day 7 and day 13 (Figure 4); however, all three genes showed an abundance of transcripts during days 25 to 33 of culture (Figure 5). This latter time interval is a stage when as many as 20%

of the vegetative shoots are preparing to initiate floral meristems (see "Experimental System"). Root TCL explants were examined only at a late stage of culture (days 25 to 33) when each explant contained several roots 1 cm or greater in length. Only low levels of FB7-1, FB7-2, and FB7-5 transcripts were detected in these root explants (Figure 5).

FB7-1, FB7-2, and FB7-5 gene expression in TCL explants was also examined by in situ hybridization. We found that, in FB7 explants, the expression of these genes was not restricted to the region of floral meristem initiation in the explant and were expressed in many cell types along the length of the explant (data not shown). FB7-1 showed more activity in cells located at the floral end of FB7 explants, but the expression of FB7-2 to FB7-5 was uniformly distributed throughout the explant.

FB7-1 and FB7-2 Genes Are Expressed in Prefloral and Floral Apices during Normal Plant Development

N. tabacum cv. samsun is a day-neutral plant in which time of flowering is related to developmental age and is not qualitatively controlled by a photoperiodic signal. The apical meristem of day-neutral tobacco passes through a number of easily recognized stages, including seedling, juvenile, adult vegetative, prefloral, and floral. The stage of meristem development can be estimated in any set of growth conditions by the number of leaves greater than 3 cm in length. The seedling meristem is small, recessed in the shoot apex, and exists until the plant has formed approximately three leaves 3 cm or greater in length. During the following period of rapid vegetative growth, the juvenile meristem increases in size and protrudes from the shoot apex. The broad, dome-shaped adult vegetative meristem then occurs just prior to the transition to flowering. Shortly after the adult vegetative stage, there is a period when the meristem becomes committed to floral development, but the shoot apex retains a morphology similar to that of the adult vegetative meristem (Waterkeyn et al., 1965; Singer and McDaniel, 1986). At this stage the meristem is pre-floral. Then follows the formation of several floral meristems that distinguish the floral apex from the vegetative and prefloral apices.

FB7-1 and FB7-2 gene expression during normal plant development was examined by in situ hybridization comparing juvenile, prefloral, and floral apical regions. Figures 6 and 7 show that transcripts for both genes were detected in the prefloral and floral shoot apices but that little, if any, transcript was found in the juvenile shoot apices. Analysis of the hybridization patterns shows that the FB7-1 and FB7-2 transcripts are localized mainly in the subapical pith cells of the immature inflorescence and are rare in the provascular cells. Thus, in both TCL explants and tobacco plants grown from seed, FB7-1 and FB7-2 transcripts are correlated with floral initiation and their appearance precedes flower organ development.

Patterns of FB7 Gene Transcripts during Normal Plant Development

Figure 8 diagrams the plant tissues used in RNA gel blot analyses to characterize more fully FB7 gene expression during normal plant development, and Figure 9 shows the results of these experiments. Poly(A^+) RNA was prepared from seedlings (including expanded cotyledons and roots); from internodes and leaves of juvenile plants; from internodes, leaves, and roots of plants having an immature inflorescence; and from floral branches and unopened flowers from fully flowering plants. FB7-1 transcripts were detected at significant levels in all internode and leaf samples as well as in unopened flowers, but were rare in seedling tissues (Figure 9A). FB7-2 probe detected two RNA species: one RNA of about 1300 base pairs and a second, less prevalent RNA of about 2200 base pairs (Figure 9B). The 1300-base pair FB7-2 transcript was found in internode and leaf tissues, with a gradient of higher expression in older tissues; this transcript was present in low amounts in seedlings and in unopened flowers, but occurred at a high level in floral branches (Figure 9B). The 2200-base pair transcript, which was not detected in TCL explants (see Figures 4 and 5), occurred at approximately the same low level in all tissues (Figure 9B). FB7-5 transcripts were detected at low levels in the internodes and leaves of juvenile plants and in the older leaves and internodes of plants possessing an immature inflorescence, but not in younger leaves and internodes or in seedlings (Figure 9C).

The most striking result of the experiment presented in Figure 9 was that high levels of FB7-1, FB7-2, and FB7-5 transcripts were found in the roots of plants possessing an immature inflorescence, but were rare in the roots of seedlings. The analysis of FB7 gene expression in roots was extended by using RNA gel blot analysis to measure the levels of FB7-1, FB7-2, and FB7-5 transcripts in the roots of plants at various stages of development. Figure 10 shows that the steady-state levels of FB7-1 and FB7-2 transcripts increased from the early juvenile stage through to the adult vegetative stage, and then decreased with the transition to flowering (Figure 10, A and B); FB7-5 displayed a similar, although less dramatic, rise and fall in the level of transcriptional expression (Figure 10C). Thus, FB7-1, FB7-2, and FB7-5 transcripts reach a maximum steady-state level in the roots just prior to the commitment to flowering and the formation of floral meristems.

DISCUSSION

Six different gene families (FB7-1 to FB7-6) were identified in our screen for floral initiation specific gene expression. FB7 gene transcripts were induced in TCL explants early during the floral developmental program, and were rare in TCL explants prior to culturing and in TCL explants early in the vegetative shoot developmental program induced by zeatin medium (Figures 4 and 5). Of the six gene

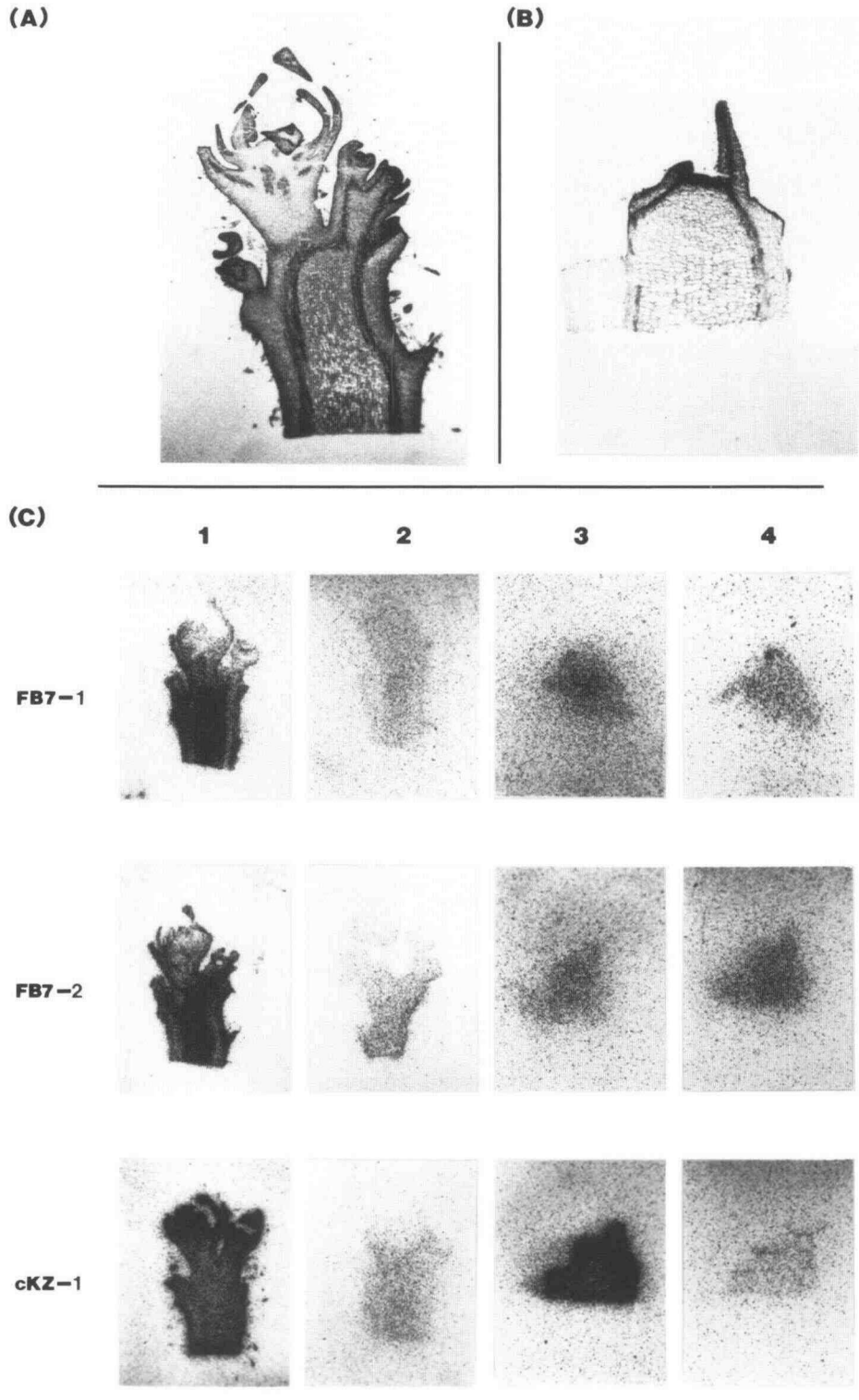

Figure 6. In Situ Hybridization to Floral and Vegetative Spices of Plants Grown from Seed.

Bright field sections of **(A)** immature inflorescence and **(B)** a vegetative apex were stained with toluidene blue. The sections of the immature inflorescence (with a well-formed terminal flower) extended approximately 12 mm into the subapical stem (**A,** magnification ×8). The sections of the vegetative apex extended about 3 mm below the meristem and leaf primordia (**B** magnification ×25). In **(C)** sections were hybridized with ^{32}P-labeled nick-translated DNA probes of FB7-1, FB7-2, and cKZ1 and exposed to Du Pont MRF 32 single-emulsion x-ray film. Images in **(C)** are bright field photomicrographs of the exposed film. Tissue sections were treated with RNase prior to hybridization as a control (columns 2 and 4). Columns 1 and 2 are sections of an immature inflorescence and columns 3 and 4 are sections of a vegetative meristem.

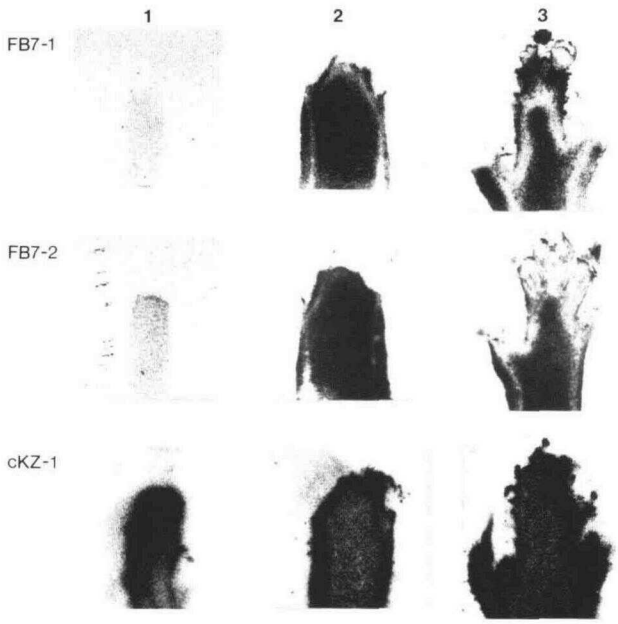

Figure 7. In Situ Hybridization to Vegetative, Prefloral, and Floral Apices of Plants Grown from Seed.

Sections of **(1)** vegetative apex, **(2)** prefloral apex, and **(3)** immature inflorescence were hybridized with ^{32}P-labeled nick-translated DNA probes of FB7-1, FB7-2, and cKZ1 and exposed to Du Pont MRF 32 single-emulsion x-ray film. Images are bright field photomicrographs of the exposed film. Tissue sections were treated with RNase prior to hybridization as a control (data not shown; see Figure 6).

families, only one, FB7-3, appeared to be kinetin-induced; the other five families did not respond to kinetin induction of vegetative shoots (Figure 3). FB7-1, FB7-2, and FB7-5 appear to be single copy or low copy number sequences in the tobacco genome, and each of these genes encodes a single major mRNA.

In floral explants the steady-state levels of FB7-1, FB7-2, and FB7-5 transcripts varied with developmental stage. The highest levels of activity were found in FB7 explants (premeristem stage) and FB25 to FB33 explants (flowering stage); FB13 explants (during meristem formation) displayed relatively low levels of FB7 transcripts (Figure 5). FB7 gene expression was at low levels during vegetative shoot formation (VS7 and VS13), but increased to high levels in VS25 to VS33 explants (Figures 4 and 5). This activity is correlated with the transition of many vegetative shoot meristems to floral meristems, supporting the implication that FB7 gene expression is related to floral initiation. In situ hybridization demonstrated that FB7 gene activity was not restricted to the region of day 7 explants immediately involved in floral meristem formation. FB7-1 expression differed from FB7-2 and FB7-5 in that its expression was more restricted to the floral end of FB7 explants. FB7-1, FB7-2, and FB7-5 gene activity is not, therefore, specific to floral meristems of TCL explants, a result which led us to examine FB7 gene expression during floral initiation in plants grown from seed.

In situ hybridization experiments demonstrated the presence of FB7-1 and FB7-2 RNAs in apices of prefloral and floral plants, but not in apices of young vegetative plants (Figures 6 and 7). The transcriptional expression is localized primarily to the subapical pith cells, with low levels of expression in other cells. Thus, during normal plant development, FB7-1 and FB7-2 transcripts occur in the subapical cells of plants committed to floral development prior to the appearance of floral organ primordia, a situation similar to that observed in floral TCL explants. This apparent association of FB7 gene transcripts with the formation of floral meristems became more complex when FB7 gene expression was examined in other plant tissues.

FB7 gene transcripts were detected in stem internode segments and leaves of plants possessing an immature inflorescence, with higher levels in the older leaves and internodes (especially for FB7-2 and FB7-5; Figure 9). However, by far the highest levels of FB7-1, FB7-2, and FB7-5 gene transcripts were found in the roots. The activity of the FB7 genes in the roots reached a maximum at the stage immediately prior to the transformation of the shoot apex to its reproductive state. FB7 transcripts were rare in 14-day-old seedling roots, increased to their highest levels in the roots of adult vegetative plants, and then decreased in the roots of plants possessing an immature

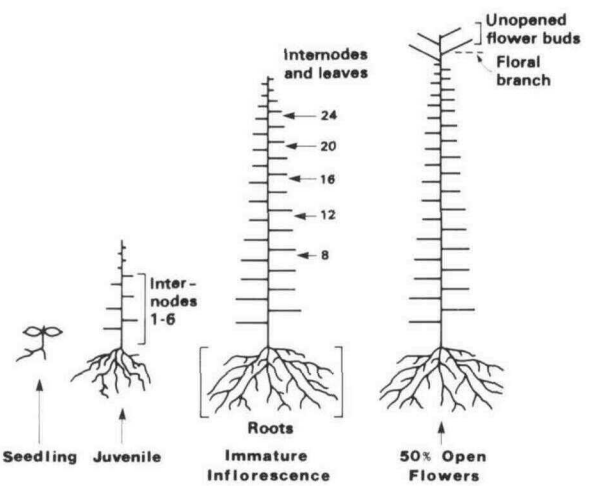

Figure 8. Diagram of Tobacco Organs Used for RNA Gel Blot Analysis.

Poly(A$^+$) RNA was prepared from 14-day-old seedlings (including expanded cotyledons and primary roots); internodes 1 through 6 (combined) from young vegetative plants; the roots, internodes 8, 12, 16, 20, and 24, and leaves 8, 12, 16, 20, and 24 from plants possessing an immature inflorescence and having produced approximately 34 nodes including the terminal flower; and floral branches and the unopened flower buds (cut at the base of the receptacle) from plants possessing a mature inflorescence.

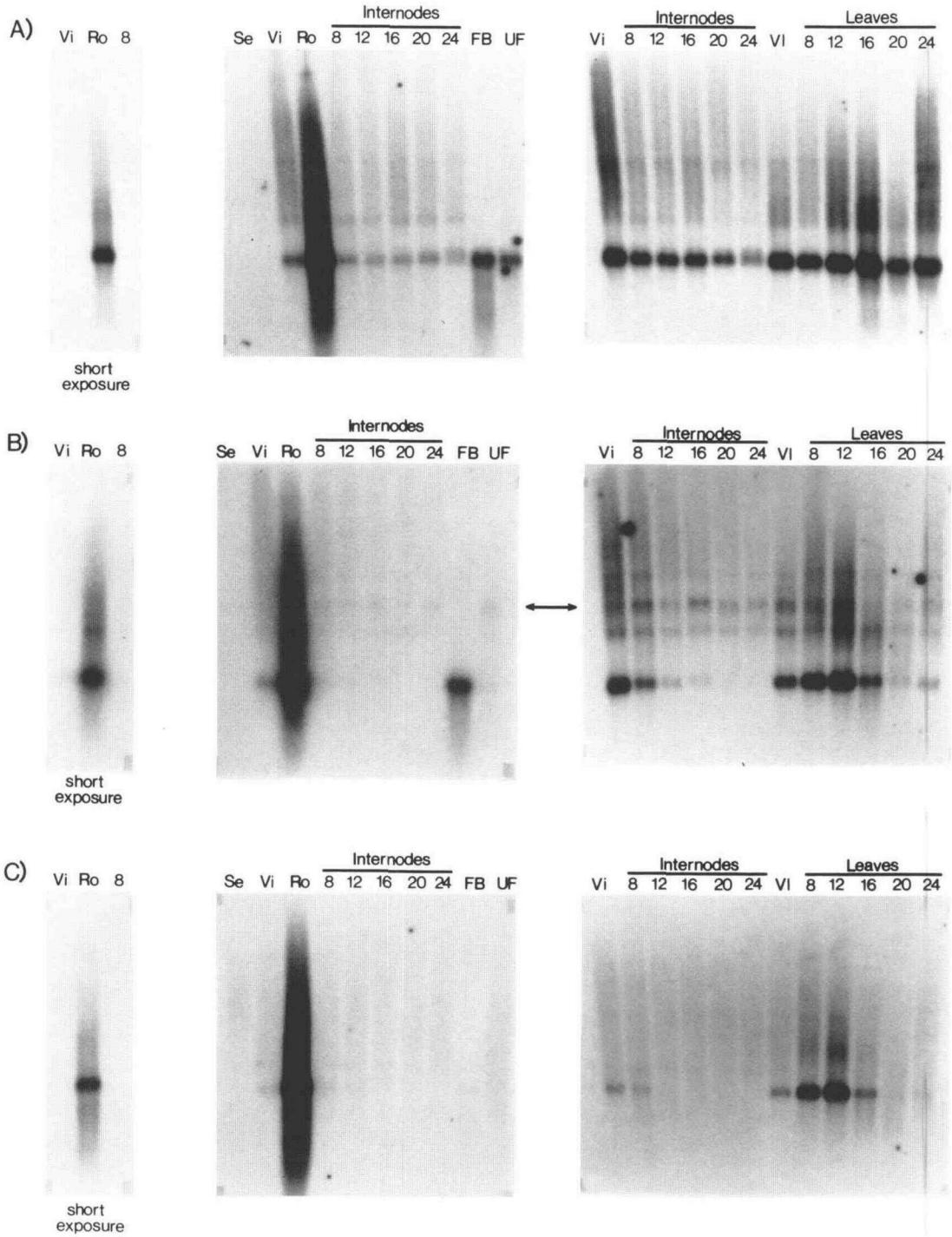

Figure 9. RNA Gel Blot Analysis of FB7 Transcriptional Expression in Tobacco Organs.

Poly(A^+) RNA was isolated from the tobacco organs represented in Figure 8. Equal amounts of RNA (2.0 μg) were fractionated by electrophoresis on a 1% formaldehyde agarose denaturing gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled single-strand RNA probes made from **(A)** FB7-1, **(B)** FB7-2, and **(C)** FB7-5. Se, seedling; Vi, internodes 1 to 6 from vegetative plants; VI, leaves 1 to 6 from vegetative plants; Ro, roots from plants possessing an immature inflorescence; FB, floral branches from flowering plants; UF, unopened flowers from flowering plants; Internodes 8, 12, 16, 20, 24, internode segments from plants possessing an immature inflorescence; Leaves 8, 12, 16, 20, 24, leaves from the corresponding internodes. Filters in the right-hand panels are approximately 7 to 10 $\times$ greater exposure times than filters in the center panels; hybridization signals between the panels may be compared with the internode RNA samples that were common to both gels. The arrow in **(B)** indicates the 2200 base pair FB7-2 transcript; other faint bands of hybridization evident in these exposures correspond to the positions of the ribosomal RNAs.

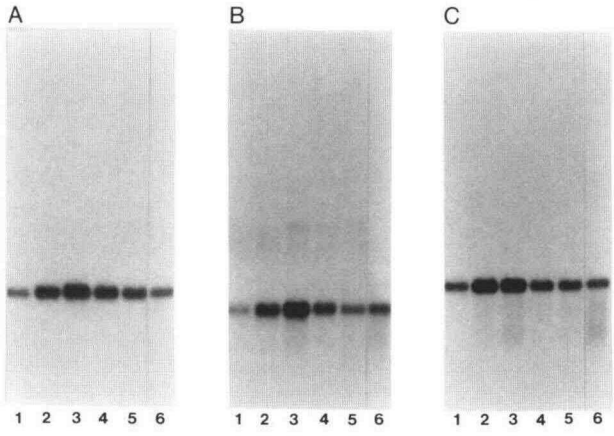

Figure 10. RNA Gel Blot Analysis of FB7 Transcriptional Expression in Tobacco Roots.

Poly(A^+) RNA was isolated from tobacco roots, and equal amounts of RNA (2.0 μg) were fractionated by electrophoresis on a 1% formaldehyde agarose denaturing gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled single-strand RNA probes: **(A)** FB7-1, **(B)** FB7-2, and **(C)** FB7-5. Roots were taken from plants at the following developmental stages: **(1)** seedling to juvenile transition, **(2)** juvenile, **(3)** adult vegetative, **(4)** pre-floral, **(5)** floral, and **(6)** immature inflorescence. The immature inflorescence RNA (lane 6) was the same RNA preparation used in the RNA gel blots of Figure 9 (lanes Ro). Densitometer scanning of the autoradiographs demonstrated an eightfold increase in hybridization intensity between lanes 1 and 3 for FB7-1 and FB7-2 (data not shown).

inflorescence (see Figures 9 and 10). That FB7-1, FB7-2, and FB7-5 gene activity was very low in day 25 to 33 root TCL explants also suggests developmental regulation of FB7 gene expression in roots.

We have described a set of genes expressed in a manner correlated with in vitro floral initiation but having activity in a number of nonfloral tissues during normal plant development. Is FB7 transcriptional activity in parts of the plants such as the subapical pith and roots consistent with these genes being involved in the early stages of flowering? To consider this question, it is important first to evaluate our use of the TCL system for isolating genes involved in flowering. A gradient of floral potential exists along the tobacco plant such that TCL explants originating from the inflorescence are capable of forming floral buds directly, whereas explants taken from more basal regions cannot produce floral buds directly, but instead produce vegetative shoots with several leaves preceding flowering. It is possible, therefore, that, at the start of culture, some cells of the floral branch-derived TCL explants have received signals while still on the plant that prepare them to initiate flowering. In such a situation gene expression specific to early floral explants may be involved not in the induction of flowering, but rather in the steps following induction (i.e. facilitating meristem formation and the development of floral organs).

To search for floral initiation specific gene expression, we compared FB7 explants with VS7 explants. Day 7 was chosen because it was well after the beginning of the TCL culture, yet preceded the organization of FB or VS meristems in the explants (see Figure 1) and should have permitted the identification gene expression involved in initiating floral organogenesis. That meristem organization occurs significantly later than day 7 may explain why the FB7 genes we isolated are not floral meristem-specific. We can speculate that expression of the FB7 genes in the subapical pith cells of prefloral and floral meristems may be related to special patterns of cell division and elongation-associated floral initiation; day 7 of culture is a period of extensive cell division in the subepidermal layer of floral TCL explants (Nassonge, 1983; D. R. Meeks-Wagner, unpublished results).

Whether FB7 gene expression in the leaves and roots is related directly to floral initiation or is involved in some parallel process cannot be answered until it is possible to block the activities of the FB7 gene products in a tissue-specific manner. However, there is substantial evidence for the involvement of both the leaves and roots in the transition from vegetative to floral development. Much of the work to date on floral induction and initiation has focused on processes occurring in the leaves. This is the result of studies on the photoperiodic control of flowering (see Evans, 1969) and on the discovery of graft-transmissible substances generated by the leaves that influence floral initiation (see Lang, 1965; Zeevaart, 1976). Such studies have led to the hypothesis that single promotive and inhibitory compounds (i.e. florigen and anti-florigen) are produced in the leaves and regulate the transition from vegetative to reproductive growth (see Bernier, 1988).

An alternative regulatory mechanism proposes that many factors are necessary for normal floral initiation (Bernier, 1986), and support for such a multifactorial model includes evidence that roots, in addition to leaves, influence the transition to flowering. Experiments with the day-neutral tobacco *N. tabacum* cv. Wisconsin 38 have demonstrated that continual rerooting of the vegetative shoot apex and upper stem perpetuated vegetative growth from the apical meristems, whereas removing the leaves from the plants had little influence on the timing of floral initiation (McDaniel, 1980). Similar results were obtained in rerooting experiments with the long-day plant *Nicotiana sylvestris* (McDaniel et al., 1985). Recent results with *Sinapis* (Lejeune et al., 1988) indicate that, by the end of the inductive long-day photoperiod, the concentration of cytokinin in root exudates increases severalfold, and it has been suggested that there may be a leaf-to-root-to-apex chain of signals generated in response to the inductive daylength. Thus, the evidence that both roots and leaves participate in floral initiation compels us to consider the possibility that FB7 gene expression in these organs is involved in floral initiation.

In this paper we have reported on the isolation of tobacco genes expressed in TCL explants during in vitro floral initiation. During normal plant development some of these genes (FB7-1, FB7-2, and FB7-5) display similar, yet complex, patterns of transcriptional activity. These expression patterns have raised the intriguing questions of how the FB7-1, FB7-2, and FB7-5 genes are regulated, and whether these three genes act to influence the same plant process. We would also like to know whether the expression of a particular FB7 gene in the roots is related to its activity in other tissues such as the shoot apex and floral branches (i.e. to determine whether a particular gene is involved in parallel plant processes). It is likely that the answers to these questions will provide insights into the relationships of various plant organs in coordinating plant growth and development.

METHODS

Plant Growth

Day-neutral tobacco *N. tabacum* cv. samsun line 5 (Tran Thanh Van et al., 1985) was the source of all plant materials. Plants were grown in a perlite/vermiculite mix in 16-hr photoperiods at 24°C day, 19°C night temperatures, and were supplemented with nutrient 3 days/week.

TCL Culture

Thin cell layers were stripped from the floral branches two to three nodes below the terminal flower; floral branches were taken from plants that had 50% open flowers. Sterilization and liquid culture of the explants were as described by Tran Thanh Van et al., 1985, except that the lighting was with the cool-white fluorescent lights at approximately 85 to 90 $\mu E\ m^{-2}\ sec^{-1}$. Floral bud (FB) culture medium was identical to Medium I described by Tran Thanh Van et al., 1985, and vegetative shoot (VS) medium was Medium I with zeatin instead of kinetin. The root TCL medium was identical to Medium I except that the concentration of kinetin was 1.25×10^{-7} M, the concentration of indole butyric acid was 7.5×10^{-6} M, and the pH was adjusted to 5.7 with potassium hydroxide.

FB and VS explants used for cDNA library construction and screening were cultured with explants distributed randomly among FB and VS culture dishes. On the 7th day of culture, explants from all but one of the FB and VS dishes were collected, frozen in liquid nitrogen, and stored at −80°C. Explants in the remaining dishes were allowed to develop until at least day 25 of culture; they were then examined for organogenesis and stored frozen at −80°C. Day 7 explants from successful cultures were pooled in order to prepare day 7 FB and VS poly(A^+) RNA. Approximately 600 each of day 7 FB and VS explants were collected.

RNA Isolation and cDNA Library Construction and Screening

RNA isolation was by the procedure of Mohnen et al., 1985. Poly(A^+) RNA was isolated using oligo(dT)-cellulose (Maniatis et al., 1983). About 100 μg of poly(A^+) RNA was isolated from each collection of 600 day 7 explants. A cDNA library containing 3.8×10^5 recombinants was made in the bacteriophage λgt10 (Huynh et al., 1985) using 5.0 μg of poly(A^+) RNA from day 7 floral TCL explants. The library was made from a population of cDNA molecules greater than 300 bp in length; size selection was by passage through a Bio-Gel A-5M column (Bio-Rad). Clones from the unamplified library were screened with ^{32}P-labeled cDNA probes. These cDNA probes were made in a standard first-strand synthesis reaction (Huynh et al., 1985) using 2.0 μg of poly(A^+) RNA with 80 units of reverse transcriptase (Life Sciences, Inc.), 1.0 mCi of ^{32}P-dCTP (3000 Ci/m; Bresa, Adelaide), 1.0 μM dATP, dTTP, and dGTP, and 0.05 μM dCTP. After 90 min at 42°C, the RNA was hydrolized with 0.1 M NaOH, and the labeled cDNA was purified from unincorporated ^{32}P-dCTP by passage over a Sephadex G-100 column. The primary screen involved 12,000 cDNA clones screened with both FB7 and VS7 cDNA probes. One hundred seventy-four clones were isolated as being FB-enriched and were checked with a second screen ("large plaque assay") in which 1.0 μl of each phage suspension (10^2 to 10^3 phage) were spotted onto a lawn of *Escherichia coli* and allowed to form plaques approximately 7 mm in diameter; duplicate nitrocellulose filters containing DNA from these recombinant phage were hybridized with FB7 and VS7 cDNA probes. Clones that were verified as having TCL floral specific gene expression were then purified as single plaques and screened a third time with FB7 and VS7 radioactive cDNA probes. This series of screens resulted in 52 cDNA clones (belonging to six different gene families, FB7-1 to FB7-6) being identified as abundant in the mRNA population of FB7 explants, but rare in the mRNA population of day 7 VS explants.

Recombinant DNA Techniques, Molecular Analysis, and in Situ Hybridization

Recombinant DNA procedures were basically as described by Maniatis et al. (1982). The cDNA inserts in λgt10 were flanked by EcoRI sites, which facilitated the cloning of cDNA fragments representing FB7-1 (1200 bp), FB7-2 (1250) bp), and FB7-5 (450 bp) into bacterial plasmids pUC19 and pGEMblue (Promega Biotec). Nick-translated probes were made using gel-purified EcoRI cDNA fragments for FB7-1, FB7-2, and FB7-5. T7 and Sp6 RNA polymerases were used to synthesize single-strand RNA probes (Melton et al., 1984) from pGEMblue plasmids containing the FB7-1, FB7-2, and FB7-5 EcoRI fragments. RNA gel blot analysis (Thomas, 1980) was performed using 1% agarose-formaldehyde gels (Maniatis et al., 1982). Single-strand FB7-1, FB7-2, and FB7-5 RNA probes were used in all RNA gel blot experiments.

In situ hybridization used cryostat cut sections of frozen apical tissue as described by Cornish et al. (1987). Frozen sections 8 to 1 μm thick were fixed at 4°C in 3.2% glutaraldehyde, prehybridized, hybridized at 42°C with ^{32}P nick-translated FB7 cDNA (approximately 1×105 cpm/μl, 3 to 8 μl/section), and washed at 42°C for 2 hr with three changes of 2 × SSC, 0.1% SDS, and 1 hr with two changes of 0.1 × SSC. Control sections were either hybridized with ^{32}P nick-translated pUC19 (data not shown), or treated with 30 μl of 1.0 μg/μl RNase at 37°C for 30 min, and then rinsed for 30 min in 200 ml of hybridization buffer (four changes), prehybridized, and hybridized with nick-translated probes. Hybridized sections were then exposed to single-side emulsion x-ray films (Du Pont MRF-32), and the images were photographed under bright field illumination.

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