

# Molecular Cloning and Characterization of Genes Expressed in Shoot Apical Meristems

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The above-ground portion of a plant develops from the shoot apical meristem. An abundant source of apical meristems was obtained from cauliflower heads. Meristematic cDNAs were identified by differential screening and used to isolate corresponding *Arabidopsis thaliana* genes. Transcriptional promoters from *Arabidopsis* clones were fused to the  $\beta$ -glucuronidase (GUS) reporter gene and introduced into plants, and GUS expression was used to analyze temporal and spatial regulation of the promoters. One promoter (meri-5) directed GUS expression in the meristematic dome and not the surrounding leaf primordia. The meri-5 promoter also directed GUS expression at branching points in the shoot and root. A second meristematic gene was found to be a histone (H3) gene. The H3 promoter was isolated and fused to GUS. Expression of the H3-GUS fusion in transgenic tobacco showed preferential expression in the peripheral zone and a lack of noticeable staining in the central zone.

## INTRODUCTION

In 1759, Wolff found that a plant's shoot originated from a localized point in the tip, the "punctum vegetations" (cited in Cutter, 1971). In contemporary terms, development of the entire above-ground portion of a plant is from a meristematic dome in the shoot apex, the shoot apical meristem. The shoot apical meristem forms the primary plant body and often undergoes differentiation to form a flower. Yet, during vegetative growth the shoot meristem functions not only to initiate new tissues and organs but also to maintain itself as a formative region.

The shoot apical meristem is the distal-most portion of the shoot apex and is typically a dome-shaped group of 800 to 1200 cells approximately 100  $\mu$ m in diameter. However, the apical meristem size varies with both species and age. Within the meristematic dome, cytological variations have led to the description of distinctive regions. A metabolically active peripheral zone initiates organs and surrounds a less active central zone (Mauseth, 1988). Although the functional significance of zonation patterns is not fully known, differentiation processes such as leaf formation are often initiated in the peripheral zone.

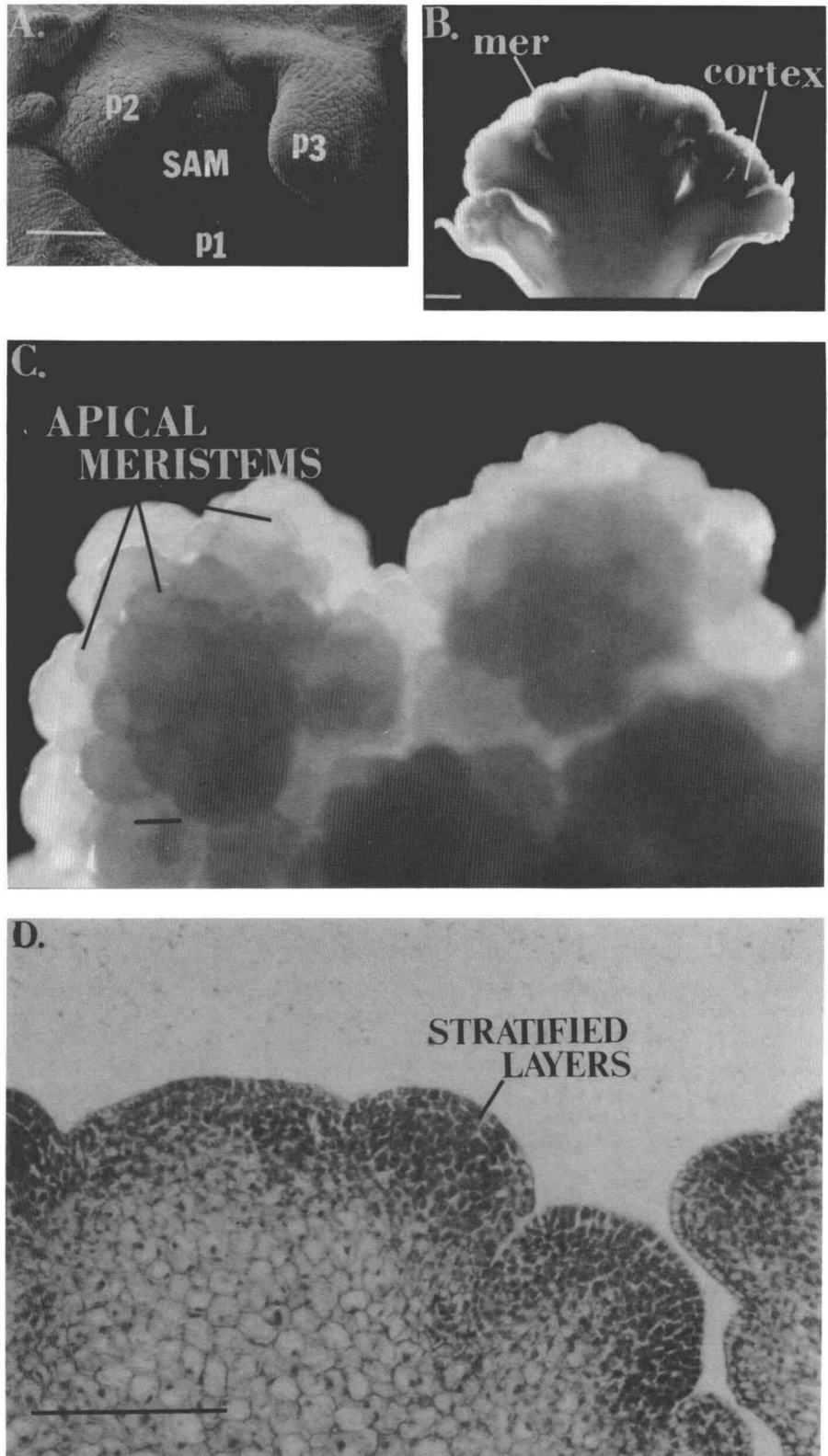
Experiments surgically disrupting the apical dome led to the suggestion that the apical meristem is an entity in itself (Sussex, 1952). Moreover, data from a number of studies show that genetic information responsible for the maintenance of the shoot apical meristem as a functional region, as well as for tissue and organ formation, is localized and

continuously expressed within the apical meristem (Barlow, 1987; Poethig, 1987; McDaniel and Poethig, 1988; Jegla and Sussex, 1989). We have initiated studies aimed at isolating genes expressed in the apical dome and deciphering how gene expression in the apical meristem functions in development of the shoot system.

The extremely small size and inaccessibility of apical meristems have hampered molecular studies of meristems. However, a *Brassica* mutation, commonly known as cauliflower, results in an enormous proliferation of apical meristems and their associated branches (De Candolle, 1824; Masters, 1869; Sadik, 1962). This proliferation leads to formation of the cauliflower head consisting of a large number of branches and  $10^5$  to  $10^6$  shoot apical meristems (Sadik, 1962; Sadik and Ozbun, 1968). The fortuitous amplification in cauliflower offers an approach to study gene expression in apical meristems.

*Arabidopsis thaliana*, a species closely related to cauliflower, has a number of features that make it important to plant biology. These features include extensive genetics, two restriction fragment length polymorphism maps, and a small haploid genome size (Meyerowitz, 1987; Chang et al., 1988; Meyerowitz, 1989; Nam et al., 1989). In addition, *Arabidopsis* has a rapid generation time and is amenable to *Agrobacterium*-mediated transformation (Lloyd et al., 1986; Valvekens et al., 1988). A major limitation of the plant is that its small size restricts the availability of tissue for some types of studies. For example, the apical meristem in *Arabidopsis* is approximately 35  $\mu$ m in diameter, among the smallest in the angiosperms (Vaughan, 1955).

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**Figure 1.** Apical Meristems in Cauliflower.

**(A)** Scanning electron micrograph of young cauliflower shoot apical meristem surrounded by three leaf primordia. The youngest primordium is designated p1 and the subsequent primordia p2 and p3. SAM, shoot apical meristem.

The similarity between *Arabidopsis* and *Brassica* extends to the DNA level. For example, a *Brassica napus* 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase gene is 92% homologous at the DNA level and 98% homologous at the amino acid level to an *Arabidopsis* gene (Klee et al., 1987; Gasser and Klee, 1990). This close relationship means that various *Brassica* species can provide complementary tools for biochemical, molecular, and histological studies in *Arabidopsis*. We have used both cauliflower and *Arabidopsis* to identify, isolate, and study genes preferentially expressed in the apical meristem. The cauliflower heads supplied apical domes in a relatively pure form and sufficient quantity to isolate and identify cDNA clones preferentially expressed in meristems. These clones were then used as probes to isolate analogous *Arabidopsis* genes so that molecular and genetic studies could be combined. Here we describe the system as a way to isolate genes from the apical meristem and report the characterization of two of these genes.

## RESULTS

### Features of Shoot Apical Meristems in Cauliflower

The shoot apical meristem is the terminating structure of the shoot and is deeply buried within leaves and leaf primordia. After removal of leaves, the meristematic dome and surrounding leaf primordia can be seen, as shown in Figure 1A. The small size of the meristem and the proximity of leaf primordia greatly obstruct isolation of the apical meristem. In cauliflower, the apical meristem is a flattened dome consisting of 800 to 1600 cells. The actual shape and size of the apical meristem depend on its point in organ initiation and the age of the plant. In the juvenile plant, the meristem is approximately 60  $\mu\text{m}$  in diameter and the upper two layers are stratified from restriction of cell division to the anticlinal plane. As the meristem enters an adult stage, the two-layer tunica stratification remains, but the dome enlarges to 180  $\mu\text{m}$  (Figure 1A).

In cauliflower, further development of the apical meristem from an adult to a floral state is arrested. Instead of a loss of zonation leading to development of floral organs, axillary meristems are initiated. The axillary meristems

themselves initiate axillary meristems that proliferate (with associated branches) to the point that the "head" is formed (Sadik, 1962; Sadik and Ozburn, 1968). The apical meristems remain terminal so that only the uppermost surface of the cauliflower head is actually meristematic (Figure 1B). Figure 1C shows that this surface consists of a multitude of meristematic domes. The remainder of the head consists of shortened bifurcated branches with enlarged and vacuolated cortical cells (Figure 1D). In addition, the distinctive stratified layers are easily recognized in apical meristems from the head, indicating that the meristems have not become determined for floral development (Figure 1D). The apical meristems found within the cauliflower head vary in size from 100  $\mu\text{m}$  to 190  $\mu\text{m}$ , depending on whether they have been recently initiated (Figure 1D).

### Identification and Isolation of cDNA Clones

The amplification in the cauliflower head provides a source of apical meristems that can be isolated in quantities. Apical meristems from immature cauliflower heads were hand harvested with the aid of a dissecting microscope. PolyA<sup>+</sup> RNA was isolated from the apical meristems and used to construct cDNA libraries (Gasser et al., 1989) that were plated and screened for clones of interest. Two independent platings and screenings of meristem cDNA libraries were done. The libraries were screened either for clones with preferential hybridization to apical meristem RNA or for clones with a high level of meristem expression.

### Identification of Clones with Preferential Expression in Apical Meristems

Because there are secondary meristems throughout the plant and potential apical meristems in the axils of every leaf, polyA<sup>+</sup> RNA isolated from the blade of mature leaves was used as a negative control for differential screening. Approximately 25,000 clones of an apical meristem cDNA library prepared in  $\lambda$ -ZAP were screened with cDNA probes. From this screening, 102 clones showing preferential hybridization to the meristem cDNA were selected. Plasmids containing the cDNA inserts from 30 of the clones were rescued according to the manufacturer's (Strata-

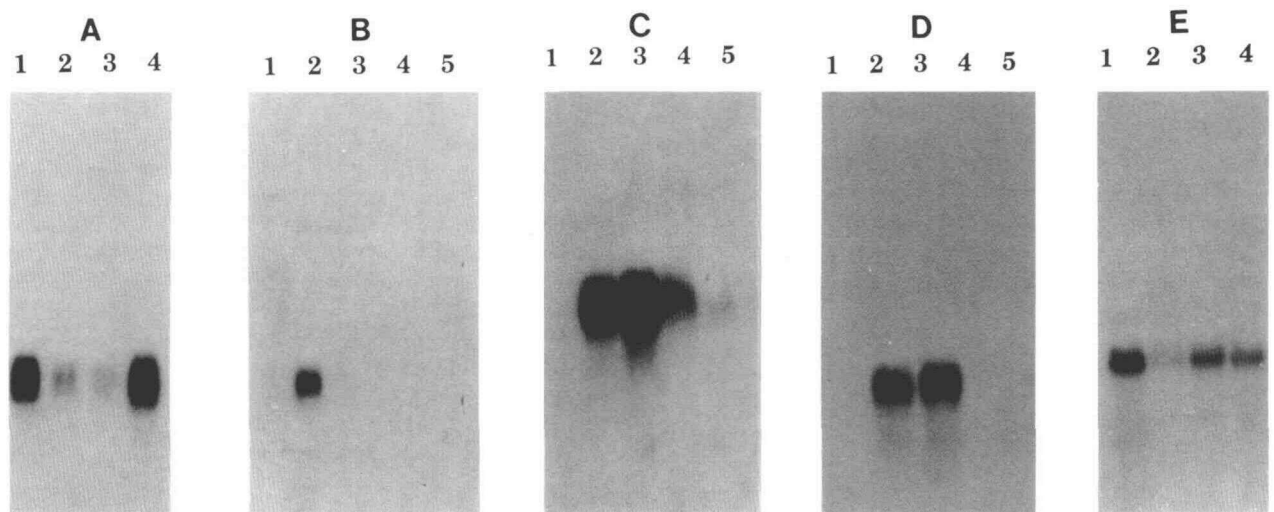
Figure 1. (continued).

(B) Free hand section through a small section of a cauliflower head. The apical domes are located in the uppermost 0.1 mm to 0.2 mm and the rest of the head consists of fused branches. Mer, apical meristems.

(C) Close-up of uppermost surface showing multiple meristematic domes.

(D) Thin section through apical meristems in the head showing the maintenance of two stratified cell layers.

Bars = 100  $\mu\text{m}$ .



**Figure 2.** Spatial and Tissue Expression Pattern of Meri Genes Analyzed with RNA Gel Blots.

(A) Meri-1 hybridized to a gel blot with 40  $\mu$ g of total RNA from 1, cauliflower apical meristems; 2, cauliflower flowers at anthesis; 3, seedlings; 4, shoot tips.

(B) Meri-2 hybridized to a gel blot with 1.6  $\mu$ g of polyA<sup>+</sup> RNA from 1, leaves; 2, meristems; 3, shoot tips; 4, seedlings; 5, flowers at anthesis.

(C) Meri-3, lanes as in (B).

(D) Meri-5 hybridized to a gel blot with 1.6  $\mu$ g of polyA<sup>+</sup> RNA from *Brassica*. 1, leaves; 2, seedlings; 3, meristems; 4, shoot tips; 5, flowers at anthesis.

(E) Meri-5 hybridized to a gel blot with 40  $\mu$ g of total RNA from *Arabidopsis*. 1, floral tissues; 2, rosette leaves; 3, stems; 4, siliques.

gene) protocol. Isolated plasmid DNAs were cleaved with a restriction endonuclease to release the cDNA inserts. The cloned DNAs were separated on agarose gels and duplicate DNA gel blots were prepared. The blots were hybridized separately to <sup>32</sup>P-labeled first-strand cDNA synthesized from apical meristem or leaf RNA. Of the 30 clones originally rescued, 19 continued to show preferential hybridization to apical meristem cDNA. Three initial clones from this screen have been characterized and more are in progress. We designated the various cDNA clones "meri" for their expression in meristems.

#### Identification of Strongly Expressed Clones from Small Gene Families

One goal of this work was to obtain a promoter conferring high transcription levels in meristematic tissue. To do this, cDNAs from low copy number genes highly expressed in meristematic tissue were isolated. These cDNAs were identified from an apical meristem cDNA library by two criteria: strong hybridization to first-strand meristem cDNA probes and weak hybridization to cauliflower genomic DNA probes. With the conditions used (see Methods), low copy number genes (less than five copies per genome) produced a weak signal with genomic probes (K. Budelier, personal

communication). One cDNA clone (meri-5), which showed an abundant hybridization signal with the cDNA probe, was isolated and analyzed further. All clones discussed showed no significant cross-hybridization to each other.

#### Tissue and Spatial Regulation of Meri cDNAs

Because the initial screenings only excluded clones expressed in mature leaves, and genes expressed in the apical meristems may be expressed in other tissues, cDNA inserts were used to probe RNA gel blots. RNA was isolated from a variety of complex tissues. The shoot apex consists of a mixture of cells from leaf primordia, proliferating cortical tissues, and the apical meristem. Because the apical meristem forms a small part of the shoot apex, a clone whose expression is limited to the dome would be expected to have a low level of expression in RNA isolated from the shoot apex. RNA isolated from seedlings included stem tissues and rapidly expanding organs as well as inactive axillary meristems.

Figure 2A shows the expression pattern for the clone with the highest and least-specific expression, meri-1. Meri-1 hybridized to an RNA of approximately 1.1 kb and was highly expressed in total RNA from both cauliflower apical meristems and shoot tips and was also expressed

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gaattcaaaagagctaaacaacatatttctaacaataatgctcatgcatgctaataat
ttactgtaacatttaaaaaaatttaaggatggttaaacataaactgcttaccaaatgatgga
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AAACCCCTCCAA
AACACACCCACACGTCAGCCACACACAAAAGACAATGCTCTCTCTTCAAAAATATTC
TCTT
M S P F K I F F F F
CAGACTCTTCTCGTGGCGCGTTTTTCAGTGTGGGCTGCTGATTCAACACTGACGTC
AA
T T L L V A A F S V S A A D F N T D V N
CGTAGCTGGGAAATGGCCGGGGAAGATACTCAACACCGCCAGCTTCTTACTCTCTC
V A W G N G R G K I L N N G Q L L T L S
CTTAGACAATCTCTGGTTCGGTTTTCAATCCAAAACAGAGTATTTGTTGGAAAGAT
L D K S S G S G F Q S K T E Y L F G K I
TGATATGCAGATTAAGCTTGTTCGGTAACTCTGCAGGAACAGTCACAACCTTTTACG
T
D M Q I K L V P G N S A G T V T T F Y
GAGTTTATATATTTCTTTAGGAGTTTTAAGTATTTGGATTTGGTTTTTATATGAGA
CTTCACTTGACATTTTGTGTATTTGCAGCTAAAATCCGAGGATCCACTTTGGATGAG
L K S E G S T W D E
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I D F E F L G N M S G D P Y T L H T N V
TACACTCAAGTAAAGTTGACAAAGAGCAACACTCCATCTCTGGTTCGACCCCAACCGCC
Y T Q G K G D K E Q Q F H L W F D P T A
AATTCCACACTTACTCAATCCTCTGGAAACCCCTCAAGAAATCATGTAAGACAACAACTC
N E H T Y S I L W N P Q R I I
CACCTTCTGTCTACACAGCTTAAATAAACCCCTAACTAGGTTTCGATTTCTTACCCTCT
CTTATCTGTTCTGTTTTCTATCAGATTGACCGTCGATACACACCCATTAGAGAGTTTAA
A
L T V D T H P L E S L K
AATATGAGTCTCTCGGTGCTTGTTCCTCAAGAAGCCGATGAGGATGGTACGGCA
T M S L S V S C F Q R T K P M R M V R Q
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F M E R Q S D W A T K R R S W L K L I G
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L K L H S W L L T E T L R L T R N Q T P
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G I G T L K K W I Q Q A K L D S N G F R R
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TGTCCTTTACTCTTATAGATATGTATAAAAAGATGTCCTGTTTTATTGTTAAAAAAA
ATTGTTTATGATAATGATAGTCTTCTTCTCTC
    
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**Figure 3.** DNA Sequence of the *Arabidopsis* Meri-5 Gene.

The transcribed portion of the gene is indicated in capital letters. The translated gene product is shown below the nucleotide sequence. The transcription start site was determined by S1 and primer extension methods. Oligonucleotides complementary to the underlined sequences were used in the primer extension analysis. The GenBank accession number is M63166.

at a moderate level in total RNA from seedlings and flowers at anthesis. The expression of a second independent clone (meri-3) is shown in Figure 2C. The meri-3 gene hybridized to a 1.0-kb RNA that is highly expressed in polyA<sup>+</sup> RNA isolated from shoot tips, apical meristems, and seedlings. The meri-3 gene was weakly expressed in polyA<sup>+</sup> RNA from flowers and not detected in polyA<sup>+</sup> RNA from leaves. Because meri-3 is expressed in those tissues that would have proliferation processes in common, meri-3 may be representative of genes involved in cell proliferation.

Figure 2B shows that the meri-2 clone showed a strong preferential hybridization to RNA isolated from apical meristems. In an RNA gel blot with polyA<sup>+</sup> RNA, meri-2 hybridized to an 800-base RNA found almost exclusively in RNA from cauliflower apical meristems. After extensive exposure, very faint signals were detected in polyA<sup>+</sup> RNA from shoot tips and seedlings. Densitometric scanning indicated that expression of meri-2 in the apical meristem was at

least 400-fold greater than in shoot tips and 100-fold greater than in seedlings. A fourth clone, meri-5, was isolated from the second screen and hybridized to a 1.1-kb RNA found at abundant levels in polyA<sup>+</sup> RNA from cauliflower apical meristems, seedlings, and, after longer exposures, flowers (Figure 2D). The lack of detectable expression of meri-5 in shoot tips is distinct from that of meri-3, whose expression was found in shoot tips as well as seedlings and apical meristems. Because the meristematic dome forms only a minor part of the shoot tip, it may not be represented in enough quantity in shoot tips for detection of meri-5 RNA. Analysis of the spatial regulation of meri-5 showed expression to be limited to specific regions of the shoot tip (see below).

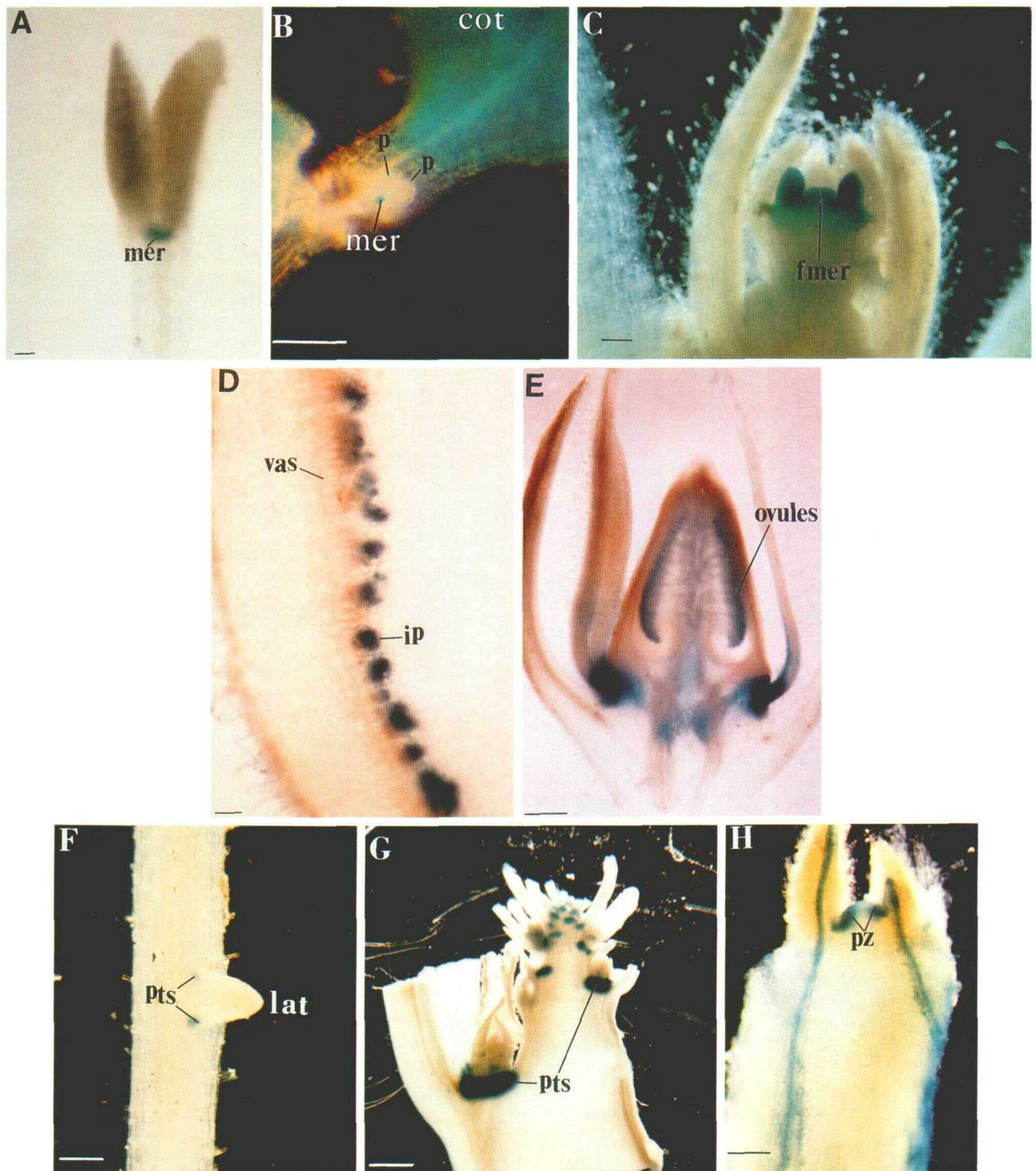
The close taxonomic relationship between cauliflower and *Arabidopsis* suggests that gene expression and function will be conserved. To compare the similarity of meri gene expression between cauliflower and *Arabidopsis*, a clone showing an abundant expression (meri-5) and a clone showing a strong preferential expression (meri-2) were used to probe an *Arabidopsis* RNA gel blot. Meri-1 was not used for these experiments because of the information from sequence analysis (see below). Figure 2E shows that with the cauliflower cDNA as a probe, meri-5 RNA was detected in *Arabidopsis* stems, floral tissues, and siliques. This indicated that the expression pattern in both *Arabidopsis* and cauliflower is similar. Meri-5 was readily detected in RNA from cauliflower seedlings that consists mainly of stem tissue. In *Arabidopsis*, meri-5 was also found to accumulate in floral stems and floral tissues. Floral tissues include flowers from bud stage to anthesis as well as pedicels. When the same RNA gel blot was probed with meri-2 cDNA, expression could not be detected (a positive control included with the hybridization was detected), presumably because of the minor contribution of the meristem in the starting material.

**Analysis of the Meri Genes**

DNA gel blot analysis using cDNA inserts from the meri-1, meri-2, and meri-3 cauliflower clones indicated that they are represented in the *Arabidopsis* genome as small gene families, whereas meri-5 is present in the *Arabidopsis* genome as a single copy gene (data not shown).

To take advantage of the genetics and small genome size of *Arabidopsis*, the cauliflower cDNA clones were used as probes to isolate corresponding *Arabidopsis* genomic clones. Hybridizing restriction fragments were subcloned, and Figure 3 shows the *Arabidopsis* sequence of one clone, meri-5. For the *Arabidopsis* genomic clone, the transcription start site was located by both primer extension and S1 nuclease analysis to a point 60 nucleotides 5' to the start of translation (data not shown). The intron and exon sequences were identified by comparison of *Arabidopsis* cDNA and genomic sequences. When the *Arabi-*





**Figure 4.** Expression of GUS Fusions to Meristem Promoters in Transgenic Plants.

- (A) GUS expression of *meri-5* localized to the apical meristem in a 10-day-old tobacco seedling.
- (B) Expression of *meri-5* GUS in the meristematic dome of 10-day-old *Arabidopsis* plants. The first pair of leaf primordia are visible.
- (C) *Meri-5* GUS expression in floral meristem and recently initiated stamen primordia in transgenic tobacco.
- (D) Cross-section of a tobacco stem showing *meri-5* GUS expression in the internal phloem.
- (E) Longitudinal section of pre-anthesis tobacco flower showing *meri-5* expression in vascular tissues leading into the flower and ovules. Strong expression was also detected at the base of the petals on the uppermost part of the receptacle.

*dopsis* coding sequence was compared to the GenBank data base, no homologous DNA sequences were found. The sequence contains one large open reading frame (ORF) of 267 amino acids in length that is presumably the meri-5 gene product. This ORF shows similarities at the amino acid sequence level to a  $\beta$ -glucanase protein from *Bacillus subtilis* (Murphy et al., 1984). However, this ORF does not show significant homology to any of the identified plant  $\beta$ -glucanases. At this time, it is not known whether the homology is significant.

For the meri-1 gene, cauliflower cDNA sequence determination showed 93% homology to the published sequence of an *Arabidopsis* histone gene, H3 (Chaboute et al., 1987). The meri-2 and meri-3 *Brassica* cDNA clones were also sequenced and showed no significant homology to sequences in GenBank. A description of these and other meri clones is in preparation (J. Medford and H.J. Klee, manuscript in preparation).

#### Expression of Meri- $\beta$ -Glucuronidase (GUS) Chimeric Genes in Transgenic Plants

The two goals of our work were to obtain transcriptional promoters conferring a high level of expression in meristematic regions and to begin to understand how gene expression is controlled in a plant's apical dome. To facilitate promoter manipulation, the *Arabidopsis* meri-5 and meri-1 (histone) genomic clones were engineered with restriction sites at the start of translation. For meri-5, a BglII site was introduced, whereas for meri-1, a BamHI site was introduced. Promoter fragments from the meri-5 and meri-1 clones were isolated using the engineered restriction sites and naturally occurring upstream sites. The meri-5 promoter was excised as a 2.4-kb BglII-StuI fragment, whereas the meri-1 promoter was excised as a 3.5-kb BamHI-KpnI fragment. The meri-5 promoter was fused to the GUS reporter gene (Jefferson et al., 1987) to create pMON673, and the meri-1 promoter was fused to the GUS gene to create pMON11006.

To analyze the temporal and spatial regulation of the meri promoters, the promoter-GUS fusions were introduced into plants. *Agrobacterium*-mediated transformation methods were used to introduce pMON673 (meri-5 GUS) into tobacco, *Brassica napus*, and *Arabidopsis* plants and the histone (H3)-GUS fusion (pMON11006) into tobacco (Horsch et al., 1985; Fry et al., 1987; Valvekens et al.,

1988). Expression of the promoter fragments was analyzed in transgenic plants by incubating tissue sections with a substrate for the GUS gene (Jefferson et al., 1987).

Figure 4A shows expression of the meri-5 GUS chimeric gene localized to the apical meristem of 10-day-old tobacco seedlings. Figure 4B shows localization of meri-5 GUS expression in the apical dome surrounded by non-staining leaf primordia. In addition to the meristematic dome, meri-5 GUS expression in young *Arabidopsis* plants persisted in post-embryonic cotyledons (Figure 4B). At this point in development in both species, the apical meristem is in a juvenile stage. As the apical meristem converted from a vegetative state to a floral state, GUS expression was detected in the floral meristem and recently initiated stamen primordia (Figure 4C). In a mature plant, the meri-5 promoter caused GUS to accumulate in the internal phloem, but not the external phloem (Figure 4D). After terminal differentiation of the apical meristem into a flower, strong meri-5 GUS expression was detected throughout vascular tissues of and leading into floral organs (Figure 4E). In floral tissues, strong GUS expression was observed at both the receptacle and the proximal end of the pedicel.

Meri-5 GUS was also expressed at a low level in the region at or near the root apical meristem in transgenic tobacco, *Brassica*, and *Arabidopsis* plants. However, Figures 4F and 4G show that the strongest meri-5 GUS expression in transgenic *Brassica* plants (and for all meri-5 GUS transgenic plants analyzed) was found at branching points in the shoot and root system. At branching points in the shoot, GUS expression was localized just beneath, but not in, cells with potential for forming axillary meristems. In roots, meri-5 GUS accumulated at points above and beneath the emergence of a lateral root (Figure 4F). Meri-5 GUS expression at these branching points in both the shoot and root system does not correspond to any known cytological differentiation. Expression of the meri-5 GUS construct showed no qualitative difference in numerous independent transgenic plants generated (see Methods for details). The consistent expression of the meri-5 chimeric gene in three species suggests that the *cis*-acting elements are conserved.

The H3 promoter-GUS fusion was also analyzed in transgenic plants. Expression of the chimeric GUS gene was observed in the vascular cambium (a secondary meristem) and at a low level throughout the shoot apex. However, Figure 4H shows that within the dome of the apical meristem, there was a distinctive pattern localized

Figure 4. (continued).

(F) Meri-5 GUS expression at branching points in *Brassica* roots.

(G) Expression of meri-5 GUS at branching points in a transgenic *Brassica* plant.

(H) Meri-1 GUS (pMON11006) expression within the peripheral zone of a tobacco apical meristem.

Bars in (A) to (D) and (H) = 100  $\mu$ m. Bars in (E) to (G) = 1 mm. Abbreviations are mer, apical meristem; cot, cotyledon; fmer, floral meristem; vas, vascular tissues; ip, internal phloem; pts, branching points; lat, lateral root; pz, peripheral zone.

to the peripheral zone in transgenic tobacco. The central zone of the meristem did not exhibit noticeable staining, supporting the idea that this zone has a reduced rate of cell division (Cutter, 1971).

## DISCUSSION

The persistence of development in plants beyond embryogenesis can be traced to small meristems. In the shoot apex, the meristem typically forms a dome-shaped structure that is the origin of the entire above-ground portion of the plant. Two recent reports that focused on meristem genes involved in the vegetative to floral transition have both described the problem of sufficient material for analysis (Kelly et al., 1990; Melzer et al., 1990). We were able to circumvent this problem by using the fortuitous amplification of material from the *Brassica* cauliflower mutation. The proliferation occurs as the meristem begins to convert from a vegetative state to a reproductive state. The apical meristem in cauliflower does not complete this conversion, but instead begins proliferating axillary meristems. Because most cauliflower cultivars need vernalization for progression to a floral state (Sadik and Ozbun, 1968, 1969), there is at least one additional step before the onset of reproductive development. In the cultivar used (Snow Crown), most apical meristems never become floral, but instead progress to a point where starch accumulates, leading to developmental arrest.

Reiteration of developmental events has been observed in a number of systems (Finkelstein and Crouch, 1984). A tomato mutation named cauliflower shows a similar proliferation of apical meristems (Paddock and Alexander, 1952). Although the tomato mutation has been lost (C. Rick, Tomato Genetics Cooperative, personal communication), in the *Brassica* genus there are many varieties maintained for agronomic purposes that represent mutations in developmental pathways. For example, similar reiterative mutants of *Brassica oleracea* include broccoli (proliferation of floral meristems) and brussel sprouts (axillary buds). The combination of these mutations as sources of materials with the molecular genetics of *Arabidopsis* provides many attractive systems for study. Furthermore, because selection of mutations (for agronomic utility) has been an ongoing process that greatly exceeds any laboratory mutational screen, it is probable that many other mutations of potential use for molecular and biochemical studies have been maintained.

Because the meristematic dome functions to proliferate cells as well as to initiate new tissues and organs, genes expressed in meristems may also be expressed in other tissues. The initial RNA gel blot analysis suggests that two of the first clones analyzed (meri-3 and meri-5) are expressed in tissues other than the apical meristem. Meri-3 was abundant in seedlings, meristems, and shoot tips,

whereas meri-5 was abundant in seedlings and meristems but not shoot tips. Because the tissues in which meri-3 was expressed are all rapidly developing tissues and apical meristems are involved in proliferative as well as formative processes, the function of the meri-3 gene product may be related to cell proliferation. In this regard, when the histone gene was hybridized to the same RNA gel blot as meri-3 (Figure 2C), it produced a similar expression pattern with stronger expression in the shoot tip and stronger expression (approximately threefold) overall. The meri-3 expression pattern, and perhaps its function, is distinct from that of meri-5. Meri-5 did not produce a detectable RNA isolated from shoot tips. When analyzed by the more sensitive assay of the promoter-GUS fusion, meri-5 was expressed only in specific regions of the shoot tip.

Expression of the promoter-GUS fusions in transgenic plants allowed us to examine the spatial and temporal expression directed by the 5' chromosomal region of two of the meri genes. In transgenic tobacco and *Arabidopsis* containing the meri-5 promoter, GUS expression was found in the meristematic dome and not in adjacent primordia. The restriction of GUS expression from the meri-5 promoter to the meristematic dome, and not the surrounding primordia (Figures 4A and 4B), provides molecular evidence supporting the view that the apical dome is an entity itself (Sussex, 1952). Expression of the meri-5 GUS fusion in the meristematic dome continued through the conversion to the floral state. In this regard, meri-5 may be a member of a general class of genes that are expressed in vegetative and floral apices (Kelly et al., 1990).

As the apical meristem (and the entire plant) developed to an adult phase, meri-5 GUS expression was detected in other regions. Expression of meri-5 GUS was also found in the internal phloem and vascular tissues leading into the flower. It is interesting to note that expression was in the internal phloem and not the external phloem. The internal phloem has been defined previously by position. In tobacco, the internal phloem is less involved in translocation of labeled compounds than the external phloem (Fahn, 1982). However, the functional significance of the internal phloem as opposed to the external phloem is not fully known. The pattern of GUS expression suggests that the internal phloem may have distinct molecular and biochemical specializations.

Meri-5 GUS expression was also found at branch points in both the shoot and root. Expression in these regions is not correlated with any known cellular differentiation. Because this expression was observed at branch points in both the shoot and root systems, there may be a function common to the emergence of organs from the primary plant body. Furthermore, because expression from the meri-5 promoter in the apical meristem and branching points was at equivalent positions in species as diverse as tobacco and *Arabidopsis*, the *cis*-acting elements directing expression for these regions must be well conserved.



The meri-1 cDNA was found to correspond to a histone H3 gene. In transgenic plants containing the histone-GUS fusion, there was a low level of expression throughout the shoot apex similar to that reported from in situ hybridizations using a clone for elongation factor 1 $\alpha$  (Pokalsky et al., 1989). The localization of the H3 expression to the periphery of the dome provides molecular evidence for zonation that was previously described on cytological characteristics. Moreover, it supports the hypothesis that the rate of cell division varies, depending on the position in the apical meristem. Earlier studies following changes with tritiated thymidine have produced conflicting answers about cell division patterns within the meristem (Steeves et al., 1969; Steeves and Sussex, 1989). The functional significance of the zonation patterns in apical meristem is not fully understood, and the meri-1 gene should prove to be valuable in understanding how cell division changes in relation to organ formation.

Although the meri genes analyzed to date are not specific for the meristem, their promoters must contain information that directs transcription in these regions. Furthermore, because meri-1 and meri-5 show different patterns of expression within the apical meristem, there are probably multiple *cis*-acting transcriptional elements that control expression in meristematic domes. Identification of the elements in the meri promoters should allow us to identify the regulatory factors that control meristematic growth. In support of this, we have already found that a specific fragment from the meri-5 promoter is retarded in gel assays when it is combined with nuclear extracts from meristems, but not from leaves (B. Xu and J. Medford, work in progress). The meri genes and tissue available from cauliflower, combined with available *Arabidopsis* mutants, will be useful tools to study how the shoot apical meristem functions in organ initiation and maintenance as a formative region.

## METHODS

### Plant Materials

For the isolation of meristematic domes, cauliflower plants (*Brassica oleracea* var botrytis cv Snow Crown) were grown in a growth chamber or freshly harvested from a local farm. When the cauliflower head reached 6 cm to 8 cm in diameter (before the onset of starch accumulation and developmental arrest), material was harvested. The upper 0.1 mm to 0.3 mm of the head was collected and the tissue was immediately frozen on dry ice. *Brassica* shoot tips were harvested from the apices of young plants and consisted of five to nine young leaves/primordia, the shoot apical meristem, and proliferating cortical cells. For seedling tissue, the shoot apex was removed from 2-week-old to 3-week-old plants, and the remaining tissue, consisting of expanding leaves and stems, was harvested. *Brassica* floral RNA was prepared exclusively from flowers at anthesis.

*Arabidopsis thaliana* tissues were harvested from plants 3 weeks to 6 weeks old. Leaves were harvested from the basal rosette, and siliques from a point midway in embryonic development. Stem material was harvested from bolted floral stems. Floral tissues consisted of open flowers, floral buds, pedicels, and the tip of the raceme.

Microscopy and histology on shoot apical meristems was done with standard botanical techniques (Sass, 1958; Jensen, 1962).

### RNA Isolation, cDNA Cloning, and Library Screening

RNA was isolated using the LiCl precipitation as described (Rochester et al., 1986). PolyA<sup>+</sup> RNA was isolated from total RNA using oligo(dT) column chromatography (Sambrook et al., 1989). cDNA cloning was done as described (Gasser et al., 1989) with the following modifications. After first-strand and second-strand cDNA synthesis and methylation, the cDNA was purified using G-50 column chromatography. Removal of unattached linkers and cDNA size selection were done with agarose gel electrophoresis. cDNA greater than 500 bp in length was collected using DEAE paper. The cDNA was eluted from the paper using high-salt buffer as directed by the manufacturer (Schleicher & Schuell). The cDNA was collected by ethanol precipitation from 0.3 M sodium acetate and washed with 70% ethanol. The cDNA was ligated into EcoRI-digested arms of  $\lambda$ gt10 or  $\lambda$ -ZAP (Stratagene) and packaged into bacteriophage using Gigapack extracts (Stratagene). Packaged  $\lambda$ gt10 phage were plated on BB4 cells,  $\lambda$ -Zap packaged phage were plated on XL-1 cells, and the percent recombinants were estimated at 80% by incubation with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside and isopropyl- $\beta$ -D-thiogalactopyranoside). Approximately 25,000 unamplified clones from each library were separately plated at low density and transferred to nitrocellulose. The library was screened as described (Gasser et al., 1989) except that oligo d(pT) was the only primer used. Clones of interest initially identified from the hybridization were subjected to further screening. The cloned cDNAs were either isolated and subcloned from the  $\lambda$ gt10 phage into plasmid vectors or rescued from the  $\lambda$ -ZAP vector using a helper phage as described by the manufacturer (Stratagene). Plasmid DNA was prepared and digested with EcoRI to release the cDNA insert. The DNA was separated through agarose and transferred to nylon (Micron Separation, Inc., Westborough, MA) membrane using standard protocols (Sambrook et al., 1989). Duplicated DNA gel blots were separately hybridized with first-strand cDNA probes using the condition previously described by Gasser et al. (1989).

### Screening cDNA Libraries for Members of Multigene Families

Duplicate filters from the apical meristem cDNA libraries were probed with cauliflower genomic DNA labeled with <sup>32</sup>P using the random primer method (Feinberg and Vogelstein, 1984). Hybridization was done using nonstringent conditions to allow cross-hybridization with multigene family members. The filters were prehybridized and hybridized in a solution containing 6  $\times$  SSPE, 5  $\times$  Denhardt's solution, 0.1% SDS, 100  $\mu$ g/mL sheared salmon sperm DNA, 20 mM Tris, pH 7.5, and 20 mM EDTA. After 48 hr of hybridization, the blots were washed at room temperature two times in 2  $\times$  SSPE, 0.1% SDS, and once at 42°C in the same solution.

### RNA Gel Blots

RNA was electrophoresed through formaldehyde gels containing 2  $\mu\text{g}/\text{mL}$  ethidium bromide and blotted onto GeneScreen-Plus (DuPont) with  $20 \times$  SSPE (Sambrook et al., 1989). The membranes were probed with cloned cDNA inserts labeled with the random oligonucleotide priming method (Feinberg and Vogelstein, 1984). RNA measurements were determined with absorbance at 260 nm and verified with ethidium bromide staining. RNA gel blots were quantified using an LKB densitometer.

### DNA Sequence Analysis and Mapping the Meri-5 Transcript Using Primer Extension and S1 Nuclease

DNA sequences were determined using synthetic primers and the Sequenase enzyme according to manufacturer's instructions (United States Biological).

The start site of the meri-5 transcript was determined by primer extension (Sambrook et al., 1989). Two separate primers complementary to the underlined sequence of Figure 3 were used. These primers were labeled with  $^{32}\text{P}$ -ATP using polynucleotide kinase and added to an extension reaction containing 20  $\mu\text{g}$  of total RNA from *Arabidopsis* floral tissue. The products were electrophoresed on a 6% acrylamide gel with a sequence ladder of the 5' end of the meri-5 gene made using the primer closest to the 5' end of the transcript. Extension products were visualized by gel autoradiography.

The meri-5 transcript start site was also determined by S1 nuclease protection (Berk and Sharp, 1977). The S1 probe was a 555-bp fragment extending from a HindIII site within the meri-5 coding sequence (Figure 3) to an upstream EcoRI site. The probe was labeled at the HindIII site with  $^{32}\text{P}$ -ATP using polynucleotide kinase. The S1 nuclease-protected fragments were resolved on a 6% acrylamide gel with  $^{32}\text{P}$ -labeled  $\phi\text{X174}$ -HaeIII size markers and visualized by autoradiography.

### Screening for *Arabidopsis* Genomic Clones

Construction of the *Arabidopsis* genomic library has been described previously (Klee et al. 1987). The library was screened with the *Brassica* cDNA clones using standard protocols (Sambrook et al., 1989). Filters were hybridized overnight with  $^{32}\text{P}$ -labeled cDNA inserts in a solution of  $6 \times$  SSC,  $5 \times$  Denhardt's solution, 0.1% SDS, 200 mM Tris, pH 7.5, 2 mM EDTA, 100  $\mu\text{g}/\text{mL}$  sheared salmon sperm DNA, and 20  $\mu\text{g}/\text{mL}$  polyA. The filters were washed three times in  $2 \times$  SSC, 10 mM EDTA, 0.1% SDS for 20 min at room temperature and once for 20 min at 50°C.

### Construction of Plant Transformation Vectors

To construct pMON673, a 3.3-kb fragment of *Arabidopsis* genomic DNA corresponding to the 5' end of meri-5 was cloned into pUC118 for manipulation. A BglII site was introduced at the translation start by site-directed mutagenesis (Kunkel, 1985), and a 2.4-kb promoter fragment was excised as a BglII-StuI fragment. The promoter fragment was ligated into BglII-SmaI-digested pMON881 (M. Hayford and H. Klee, unpublished data) to create pMON672. A 2.2-kb HindIII fragment from pMON607 (Medford et al., 1989) containing the GUS gene fused 3' to a nopaline synthase polyadenylation signal was excised and ligated into pUC8 creating pMON671 to obtain BamHI sites for subsequent ligation. The 2-kb GUS gene from pMON671 was excised independent of the

nopaline synthase 3' end with BamHI. This fragment was ligated into BglII-digested pMON672 to create pMON673.

To construct pMON11006, a 5.9-kb genomic clone containing the promoter of the *Arabidopsis* H3A725 (Chaboute et al., 1987) gene was engineered to contain a BamHI site at the start of translation. A 3.9-kb KpnI-BamHI promoter fragment was cloned into KpnI-BglII-cut pMON607 (Medford et al., 1989), creating an H3-GUS transcriptional fusion, pMON11006.

### Plant Transformation

Plasmids were mobilized into *Agrobacterium* ASE (Fraley et al., 1985) using a triparental mating system (Ditta et al., 1980). *Nicotiana tabacum* cv Xanthi plants transformed with pMON673 and pMON11006 were obtained using the leaf disc method (Horsch et al., 1985). Plasmid pMON673 was transformed using kanamycin selection and pMON11006 was transformed using gentamicin selection (Hayford et al., 1988). *Brassica napus* cv Westar plants were transformed using the method of Fry et al. (1987). *Arabidopsis* ecotype RLD plants were transformed using the root method (Valvekens et al., 1988) with the following modifications. Carbenicillin (750 mg/L) was used to eliminate the *Agrobacterium* after a 2-day coculture. In addition, because carbenicillin has a weak auxin effect, auxin levels in the shoot-inducing media were reduced to 0.1 mg/L. Progeny from the primary transformants were identified by germinating seed on medium containing 50  $\mu\text{g}/\text{mL}$  kanamycin (tobacco and *Arabidopsis*) or by GUS activity. There was no qualitative difference in meri-5 GUS expression when analyzed in 20 independent transgenic tobacco plants, five independent transgenic *Arabidopsis* plants, and three independent *B. napus* plants. Similarly, there was no qualitative difference in four independent tobacco plants containing the H3-GUS (pMON11006) fusion.

### Histological Analysis

Transgenic plants were sectioned by hand and the cut surface was immediately placed in a buffer containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide as described (Jefferson et al., 1987) except that the solution contained 20 mM sodium sulfite as an anti-oxidant. Sections were incubated at 37°C for 4 hr to overnight. The tissues were fixed and pigments removed by placing the tissues in either 70% ethanol or formalin:ethanol:water:acetic acid (2:8.5:8.5:1). The cut sections were photographed using a Zeiss stereomicroscope.

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