The Plant Cell, Vol. 10, 877–887, June 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

RESEARCH ARTICLE

# Transposon Tagging of the *Defective embryo and meristems* Gene of Tomato

**James S. Keddie,[a,1,2] Bernard J. Carroll,[b,1] Colwyn M. Thomas,[c] Melquiades E. C. Reyes,[b] Victor Klimyuk,[c] Hans Holtan,[a] Wilhelm Gruissem,[a] and Jonathan D. G. Jones[c]**

[a]Department of Plant Biology, 211 Koshland Hall, University of California, Berkeley, California 94720
[b]Departments of Biochemistry and Agriculture, University of Queensland, Brisbane 4072, Queensland, Australia
[c]Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom

**The shoot and root apical meristems (SAMs and RAMs, respectively) of higher plants are mechanistically and structurally similar. This has led previously to the suggestion that the SAM and RAM represent modifications of a fundamentally homologous plan of organization. Despite recent interest in plant development, especially in the areas of meristem regulation, genes specifically required for the function of both the SAM and RAM have not yet been identified. Here, we report on a novel gene, *D*efective *e*mbryo and *m*eristems (*Dem*), of tomato. This gene is required for the correct organization of shoot apical tissues of developing embryos, SAM development, and correct cell division patterns and meristem maintenance in roots. *Dem* was cloned using transposon tagging and shown to encode a novel protein of 72 kD with significant homology to YNV2, a protein of unknown function of *Saccharomyces cerevisiae. Dem* is expressed in root and shoot meristems and organ primordia but not in callus. The expression pattern of *Dem* mRNA in combination with the *dem* mutant phenotype suggests that *Dem* plays an important role within apical meristems.**

## INTRODUCTION

In plants, organogenesis is continuous and occurs in apices throughout the entire life cycle. This process is achieved by the action of apical meristems, which are groups of stem cells that are established early in embryogenesis and maintained in the tips of shoots and roots. Because apical meristems are almost entirely responsible for the elaboration of plant architecture, they have been a major subject of observational, experimental, and genetic studies (described in Steeves and Sussex, 1991; Meyerowitz, 1997). We are now beginning to elucidate the genes involved in meristem regulation and to understand their function (Meyerowitz, 1997).

In angiosperms, the shoot apical meristem (SAM) is usually a small dome of cells that consists of a peripheral zone in which leaves are initiated and a central zone in which the peripheral zone cells are replenished. The central zone contains cells that divide slowly, whereas the peripheral zone contains cells that divide rapidly (Lyndon, 1990; Steeves and Sussex, 1991). Superimposed upon this zonation are three clonally distinct cell layers (Poethig, 1987): L1 (forming the epidermis), L2 (forming the mesoderm), and L3 (forming the pith and vascular tissue). These cell layers generate the whole shoot. The L1 and L2 layers in the SAM are maintained by anticlinal cell divisions. Occasional cell divisions occur that result in the insertion of cells derived from one layer into the adjacent layer. These cells adopt a fate appropriate to their new layer, thus suggesting that positional information, rather than cell lineage, is the major factor influencing cell fate decisions during plant development. How cells in meristems communicate with each other has not yet been determined; however, recent results indicate roles for protein trafficking (Lucas et al., 1995) and extracellular signaling (Clark et al., 1997).

The root apical meristem (RAM), in contrast to the SAM, is an internal area of cells and is responsible for the production of cells for both the root and the root cap. The RAM is therefore surrounded on all sides by its derivatives. At the center of the root meristem is a region of cells known as the quiescent center—a population of cells that has a very long generation time. Surrounding the quiescent center are initial cells, which divide more rapidly and whose progeny differentiate into the basic cell types of the root and root cap. The cells of the quiescent center are proposed to act as replacements for the more rapidly dividing apical initials. Cell division patterns within the Arabidopsis root are almost invariant, which results in a root comprised of several clonally distinct

[1] These authors contributed equally to this work.
[2] Current address: Mendel Biotechnology Inc., 21375 Cabot Boulevard, Hayward, CA 94545. To whom correspondence should be addressed. E-mail jkeddie@mendelbio.com; fax 510-264-0254.

files of cells (Dolan et al., 1993). Lateral roots are not initiated at the root apex but rather are initiated from an internal layer of cells called the pericycle. Experimental evidence suggests that the root tip inhibits the formation of lateral roots (McCully, 1975).

Despite their differences, the basic organization of the SAM and RAM is similar: both meristems are layered structures that contain a central zone of quiescent or slowly dividing cells. In addition, experiments using surgically isolated meristems have shown that the SAM and RAM are autonomous in their development (Ball, 1952; Feldman and Torrey, 1976). These observations have led to the conclusion (Steeves and Sussex, 1991) that the differences between the SAM and RAM are superimposed upon a fundamentally homologous plan of organization and that the root and shoot systems probably represent evolutionary modifications of an "ancestral meristem" in response to different environments. Mutations that specifically affect both the SAM and RAM may therefore represent lesions in genes whose functions have been conserved throughout the evolution of apical meristems from the ancestral meristem.

In this study, we describe a recessive mutant of tomato, *defective embryo and meristems* (*dem*), that is affected in the development of both shoot and root apical meristems. *Dem* was cloned by using the transposable element *Dissociation* (*Ds*) as a tag and shown to encode a novel protein with a region of significant homology to a yeast protein of unknown function. *Dem* is expressed in SAMs and RAMs, axillary meristems, and organ primordia during adult plant growth. Although the exact function of *Dem* remains unclear, our initial observations suggest that it plays an important role within apical meristems and organ primordia.

## RESULTS

### *dem* Mutants Have Disrupted Apical Meristems

A total of 150 families carrying independent transpositions of the maize transposon *Ds* in tomato were generated. Approximately 25 seeds from each family were sown in flats, and seedlings were screened for mutant phenotypes. *dem* mutants were found in one family, N174 (Figure 1A). Testcross and $F_2$ analysis showed that the mutation was recessive and that mutant progeny occurred at a frequency of 10 to 15%. Self-pollination of heterozygotes revealed that the *dem* mutants had a highly variable number of small, slightly concave, abnormal cotyledons and no SAM. Wild-type seedlings were normally dicot (Figure 1B). Of 110 mutants inspected, two were monocot, 20 were dicot, 65 were tricot, and 23 were tetracot (Figures 1C to 1F).

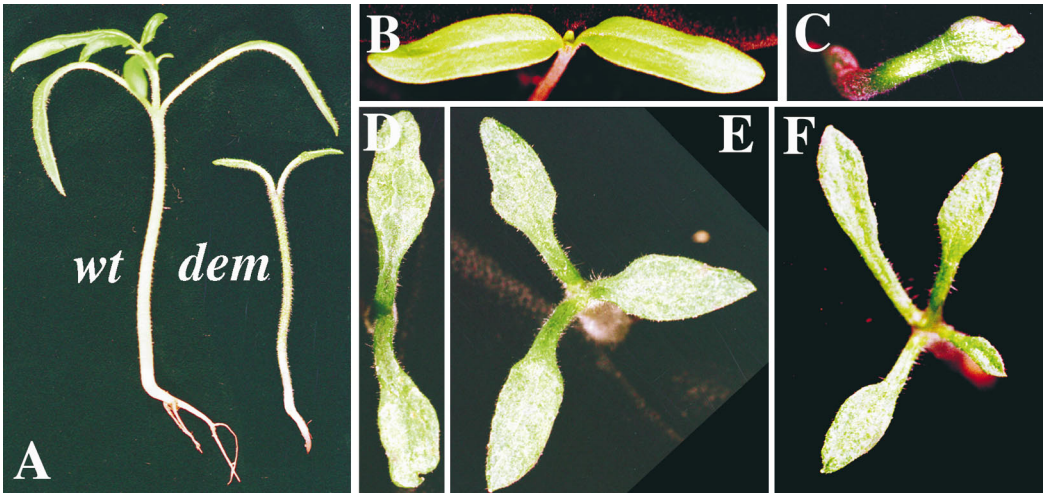


**Figure 1.** Seedling and Embryo Morphology Is Disrupted by the *dem* Mutation.

**(A)** Three-week-old *dem* and wild-type (*wt*) plants. *dem* plants have neither elongated roots nor a shoot.
**(B)** Wild-type dicot seedling.
**(C)** *dem* monocot.
**(D)** *dem* dicot.
**(E)** *dem* tricot.
**(F)** *dem* tetracot.

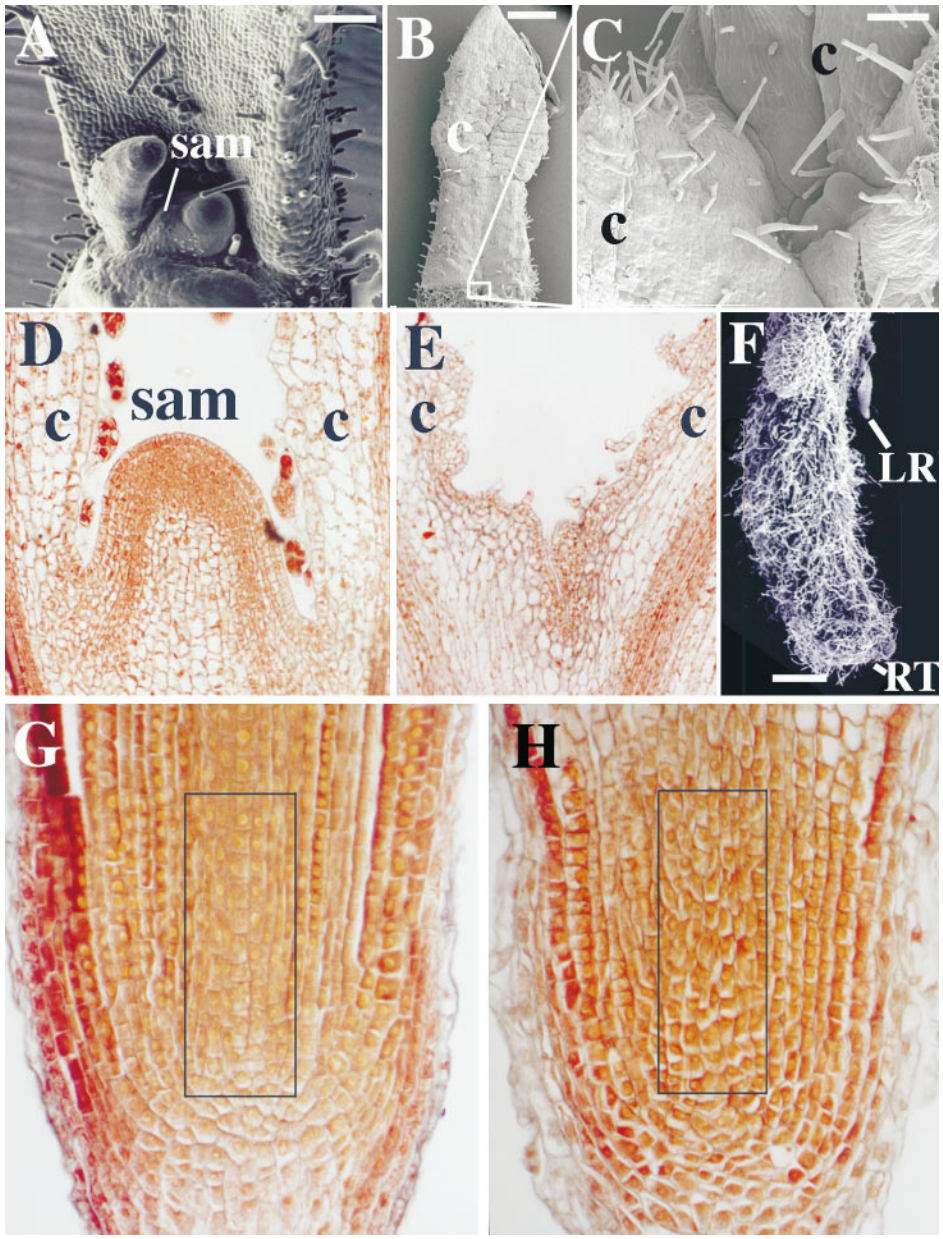


**Figure 2.** *dem* Seedlings Have No Apical Meristem.

**(A)** and **(B)** SEM of the SAM (sam) and a cotyledon (c) of a wild-type seedling and the cotyledon and shoot apical region of a *dem* mutant, respectively. One cotyledon has been cut off in **(A)** and **(B)** to facilitate viewing. Bar in **(A)** = 150 μm; bar in **(B)** = 719 μm.
**(C)** An expanded view of the *dem* apical region boxed in **(B).** Bar = 76 μm.
**(D)** Section through the wild-type shoot apex.
**(E)** Section through the *dem* shoot apex. No typical SAM can be seen. The adaxial tissues of the cotyledons are disorganized.
**(F)** SEM of a *dem* root, showing a lateral root (LR) and root tip (RT). Bar = 712 μm.
**(G)** Section through the wild-type root apex, showing a typical root meristem.
**(H)** Section through the *dem* root apex, showing that the *dem* root meristem (boxed) is disorganized.

Apical growth of *dem* seedlings was terminated soon after germination, and no true leaves were initiated (Figure 2). Scanning electron microscopy (SEM) studies (Figures 2A to 2C) showed that the apical region between the cotyledons of dicot *dem* seedlings usually contained no SAM or leaf primordia. Sections through *dem* apices (Figures 2D and 2E) confirmed that no organized SAM was present in *dem* seedlings but rather that tissue with a disorganized cell arrangement formed. This disorganization continued from the axis of the cotyledons into the adaxial half of the cotyledons. Cell organization in the abaxial half of cotyledons appeared to be normal. *dem* roots terminated after 3 or 4 mm of growth, and lateral roots, which also aborted after a short period of extension (Figure 2F), were initiated. *dem* roots were also very hairy; however, it is not possible to predict whether this is a direct effect of the mutation. Sections through a *dem* root show that although many of the outer cell files are correctly maintained, cells in the center of the root apex are disorganized compared with the wild type (boxed in Figures 2G and 2H). No clear cell files were observed in the central cylinder of a *dem* root.

### Isolation of the *Dem* Gene by Transposon Tagging

Sequences flanking the *Ds* element in a *dem* mutant were cloned using inverse polymerase chain reaction (IPCR) (Thomas et al., 1994) and sequenced. Using this sequence, two primers, dem3′ and dem5′, were designed. When used in combination with primer B34 (Thomas et al., 1994), they could be used to map the *Ds* element in relation to the *dem* phenotype. In tests of 200 individuals of a segregating population using triplex PCR, *Ds* was found to segregate with the *dem* phenotype (Figure 3A), demonstrating close linkage between the mutant phenotype and a *Ds* insertion. A transposase source, stabilized *Activator* (*sAc*), was crossed onto a *dem* heterozygote, and an $F_1$ plant containing both *sAc* and *Ds* was self-pollinated. Approximately 75% of the *dem*

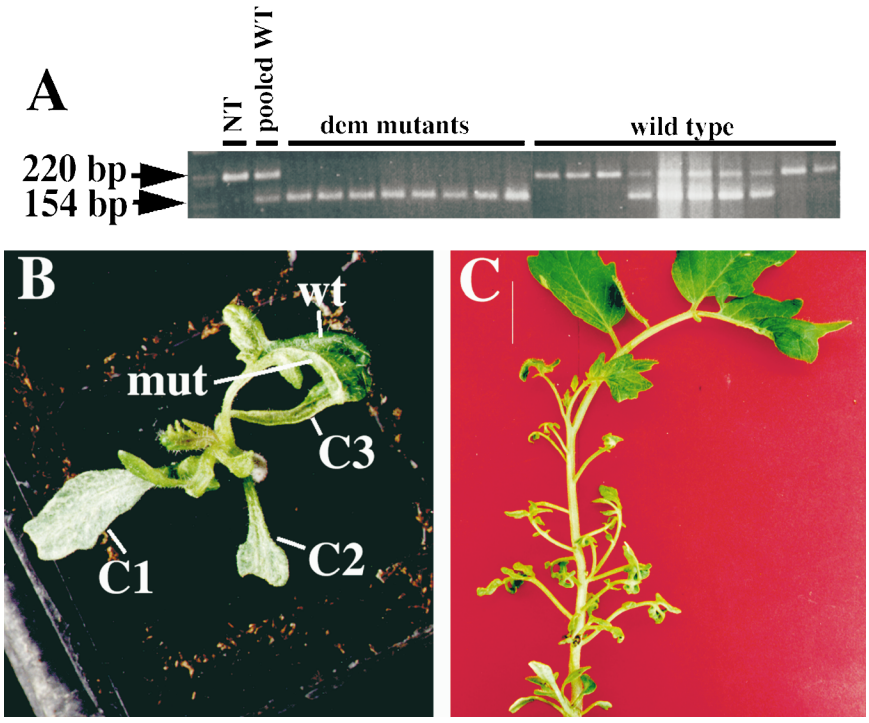


**Figure 3.** Linkage of *Ds* to the *dem* Mutation and Somatic Reversion of *dem*.

**(A)** Linkage of the *Ds* insertion to the *dem* mutation was demonstrated using a PCR zygosity test: a 220-bp fragment was amplified from the preinsertion allele, and a 154-bp fragment was amplified from the *Ds* insertion allele. PCR with DNA from stable mutant seedlings only produced a 154-bp fragment, indicating that these seedlings are homozygous for the *Ds* insertion and that the *Ds* is closely linked to the *dem* mutation. Wild-type plants were either heterozygous or homozygous for the preinsertion allele. NT, untransformed; WT, wild type.
**(B)** and **(C)** Transposase-dependent somatic reversion of the *dem* phenotype confirmed that the *Ds* insertion is the cause of the *dem* mutation. For further details, see Methods and Results. Somatic revertants also initiated leaves that could not develop properly. mut, mutant; wt, wild type; C1, C2, and C3 indicate cotyledons.

mutants in this segregating population contained *sAc.* After a period of up to 2 months, all of these mutants reverted and formed shoots from between their cotyledons. These shoots were either fully wild type or chimeric. The chimeric shoots contained both wild-type and mutant tissues and were rather unusual in appearance (Figures 3B and 3C). After a period of time, chimeric shoots became fully wild type in appearance. In contrast, *dem* mutants that did not contain *sAc* never formed shoots, even after several months. The shoots of somatic revertants yielded fruit that contained viable seed. Seeds from somatic revertants were planted, and seedlings were scored for the *dem* phenotype: the majority of these seedlings were wild type, demonstrating that the *dem* mutation is germinally unstable in the presence of the transposase gene. The close linkage of *Ds* with the *dem* phenotype and the *sAc*-dependent somatic and germinal instability of the *dem* phenotype strongly implicate *Ds* as the cause of the *dem* mutation.

An 8-bp target site duplication is typical of *Ds* insertion, and many *Ds* excision alleles retain this duplication or have deletions/substitutions of one or two nucleotides (Saedler and Nevers, 1985). To confirm that the *dem* mutation was caused by a *Ds* insertion, DNA from germinal revertants was prepared, and the sequence alterations expected from *Ds* excision were analyzed. All sequenced *Dem* revertant alleles contained sequence alterations consistent with *Ds* excision (Figure 4A). This result confirms that the *dem* mutation is a result of a *Ds* insertion into the *Dem* locus. The *Ds* insertion allele of *dem* was designated $dem^{Ds}$.

During the course of the analysis of germinal revertants, a $sAc^{-}$ $Ds^{-}$ plant was identified that gave rise to ~10% mutant progeny. This allele of *dem* was later sequenced and found to contain a 7-bp insertion at the *Ds* insertion site that causes an early frameshift in the *Dem* open reading frame (ORF). This allele was designated $dem^{+7}$. Plants homozygous for $dem^{+7}$ displayed a phenotype identical to $dem^{Ds}$, demonstrating that $dem^{Ds}$ is probably a null allele. The phenotypic analysis described above was performed with mutants homozygous for $dem^{Ds}$.

In a separate experiment, $dem^{+7}$ heterozygotes containing *sAc* were crossed onto $dem^{Ds}$ heterozygotes, and several somatic revertants were identified. DNA was extracted from wild-type tissues of these mutants. The sequences surrounding the site of *Ds* insertion were amplified by polymerase chain reaction (PCR) using oligonucleotides dem3′ and dem5′, with one being kinase labeled. PCR products were then size fractionated by PAGE. All revertant alleles represented either perfect excision events or insertions/deletions of +3, +6, or −3 nucleotides (Figure 4B). These sequence alterations restored the *Dem* reading frame and resulted in the addition or loss of one or two amino acids in the Dem protein. In one case, a deletion of 18 nucleotides (leading to a deletion of six amino acids in the Dem protein) was identified. These results are consistent with the idea that *Ds* insertion occurred in the *Dem* coding sequence and that only excision events that do not alter the reading frame will reinstate wild-type gene function. Amino acid residues around this area are therefore not essential for the function of the Dem protein.

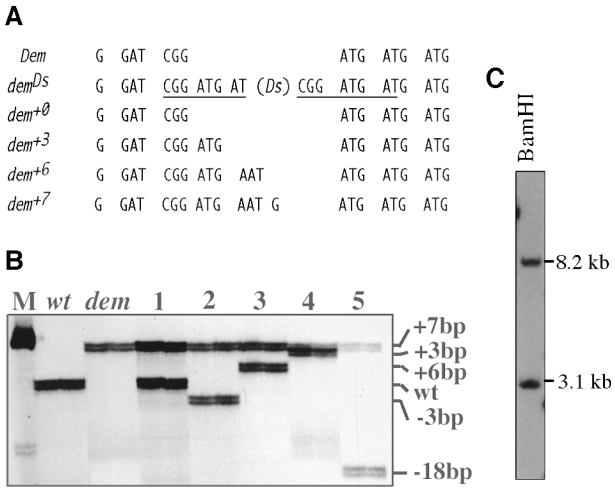


**Figure 4.** *dem* Excision Alleles.

**(A)** *Ds* insertion into *Dem* creates an 8-bp direct repeat (underlined). *dem* excision alleles containing in-frame insertions (+6 and +3) and wild-type sequence all restored wild-type gene function. $dem^{+7}$ is a stable allele of *dem* containing a 7-bp insertion. $dem^{+7}$ is predicted to produce a peptide of 123 amino acids before translation is terminated.

**(B)** Gel analysis of excision alleles, showing that footprints of −3, +3, +6, and −18 (lanes 2, 3, 4, and 5, respectively) reinstate wild-type (wt) gene activity. These revertants contain the $dem^{+7}$ mutant allele and the revertant wild-type allele. M indicates length markers.

**(C)** Blot of BamHI-digested tomato genomic DNA hybridized with the *Dem* cDNA. A BamHI restriction site exists within the *Dem* cDNA; therefore, two bands of 8.2 and 3.1 kb indicate one gene.

PCR tests showed that $dem^{Ds}$ is fully transmitted through male and female gametes. The observed segregation distortion (10 to 15% mutant rather than 25% mutant) is due to decreased viability of *dem* embryos (M.E.C. Reyes and B.J. Carroll, unpublished data). DNA gel blotting experiments using low- and high-stringency washes demonstrated that *Dem* is present as a singly copy in the tomato genome (Figure 4C).

### *Dem* Encodes a Novel Protein

Cloning and sequencing of the flanking DNA of this mutant line revealed that the *Ds* element had inserted in a large ORF. The cloned flanking sequences were used to screen a cDNA library. One full-length *Dem* cDNA clone was isolated and sequenced (Figure 5; GenBank accession number

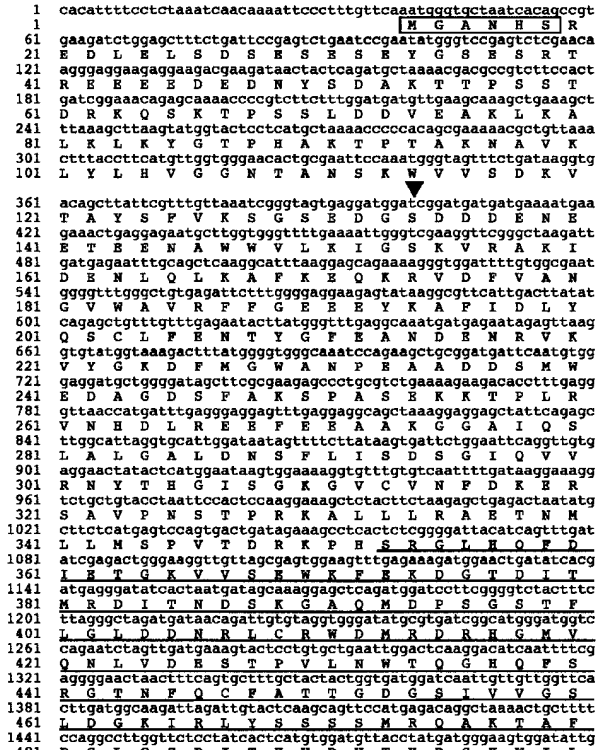

**Figure 5.** *Dem* cDNA and Deduced Amino Acid Sequence.

The DNA sequence of the longest *Dem* cDNA is shown, with the predicted amino acid sequence provided at the bottom. The region of homology to YNV2 is underlined, and a potential myristoylation motif is boxed. *Ds* was inserted into codon 121 of *Dem* in *Dem*$^{Ds}$ plants (indicated by a filled triangle). The GenBank accession number is Y13632.

Y13632). This cDNA contained one long ORF with an in-frame stop codon in the 5′ leader sequence. Translation of the ORF predicted a charged protein of 71,919 D with a pI of 5.58 (Figure 5).

A search of the PROSITE database showed that the predicted mature N-terminal sequence of Dem, MGANHS, conforms to the consensus sequence for N-myristoylation, suggesting that Dem may be attached by a lipid anchor to a cellular membrane. BLAST (Altschul et al., 1997) searches using the Dem peptide sequence identified two potentially homologous proteins (Figure 6A): CYPRO4 from artichoke thistle (GenBank accession number P40781; 93% identical and 98% similar; $P = 5.4 \times 10^{-260}$) and YNV2 from *Saccharomyces cerevisiae* (GenBank accession number P40157; 33% identical and 51% similar over 150 residues; $P = 1.7 \times 10^{-11}$). Both proteins are of unknown function. Two Arabidopsis expressed sequence tags (F19919 and N96644) with strong homology to the 3′ and 5′ ends of *Dem* were also identified (Figure 6B).

## *Dem* Is Expressed in Apical Meristems and Organ Primordia

The expression pattern of *Dem* was investigated initially using RNA gel blot hybridization analysis (Figure 7). *Dem* mRNA is expressed at high levels in shoot apices and at much lower levels in roots, young fruit, stem, mature leaves, and seedlings. Importantly, no *Dem* transcript was identified in RNA prepared from callus, suggesting that Dem is not a component of the cell cycle machinery and is not required for cell maintenance or unpatterned cell division.

*Dem* expression was further localized by in situ RNA hybridization (Figures 8A to 8M). In shoot apices, *Dem* expression was restricted to apical meristems and adaxial sides of leaf primordia (Figures 8A, 8C, and 8D) and young leaves (Figure 8F), which corresponds closely to the tissues affected in *dem* seedlings. *Dem* was downregulated in mature leaf tissue and upregulated in the adaxial side of the leaf in the region of developing leaflet primordia (Figure 8G). *Dem* was also expressed in dormant axillary meristems (Figure 8I) and in floral meristems and developing flowers (Figure 8J). In root tips, *Dem* mRNA formed a gradient that was most concentrated at the root apex (Figure 8L). Overall, *Dem* was expressed in tissues of adult plants in which organized cell division occured and in vascular strands. No signal was observed in sense strand controls (Figures 8B, 8E, 8H, 8K, and 8M).

## DISCUSSION

*Dem* is expressed in all regions of the plant in which organized cell divisions take place. These regions include apical meristems, organ primordia, and leaflet primordia. However, *Dem* is not expressed in callus. Furthermore, loss of *Dem* function causes disorganization of both the shoot and root apex and in the adaxial tissues of cotyledons. These observations suggest that *Dem* is required for the organization or maintenance of meristems and primordia. *dem* mutants are morphologically distinct from those previously reported to be affected in basic body planning (Jurgens et al., 1991; Mayer et al., 1991), SAM development (Caruso, 1968; Meyerowitz, 1997), and root development (Benfey and Schiefelbein, 1994) and may represent a novel category of mutants that are affected in a basic aspect of meristem regulation.

**A**

```
Dem 340   SRGLHQFDIETGKVVSEWKFEKDGTDITMRDITNDSKGAQMDPSGSTFLGLDDNRLCRWDMRDRHGMVQNLVDESTPV
          .. |..|||  |||..||.    ..| .: :.. ..| .||.|.. |::|:.:. : : |  |  :|. .  ||||.
YNV2 491  ENKLYKMDIERGKVIEEWS....TGDKNVVQYGPTKKFDQMTPEQ.TIVGVSQKGVFKIDPRI.NGKNKIAVDESK..

Dem 440   LNWTQGHQFSRGTNFQCFATTGDGSIVVGSLDGKIRLYSSSSMRQAKTAFPGLGSPITHVDVTYDGKWILGT 489
                ::    || :::||:.|  |.:|| .|.|:||.. ::|  ||||:|:|| :|. :... ||||:|:|
YNV2 576  .......DYVGKYNFSSIGTTESGYIAIGSEKGDIKLYDRLGIR.AKTAIPSLGQAIKFITTSADGKWLLAT 624
```

**B**

```
Dem 563       VGKFSVIWNFQQVKDGSHECYQNQVGLKSCYCYKIVL..RDDSIVESRFMHDKYAVS 599
              VGKFSVIW+ ++VK+ +HECY+NQ GLKSCYC KI++  +D+SIVESRFMHD ++ S
F19919        VGKFSVIWDLERVKNSAHECYRNQQGLKSCYCNKILVLLKDESIVESRFMHDNFSFS

Dem  56       SSLDDVEAKLKALKLKYGTPHAKTPTAKNAVKLYLHVGGNTANSKWVVSDKVTA 109
              SSLDDVEAKL+ALKLKY    +  P+ +N+ +L+ ++ GNT  +KWV ++K+TA
N96644        SSLDDVEAKLQALKLKYPLTQSA.PSTQNSARLFRYINGNTPKAKWVTAEKLTA
```

**Figure 6.** Sequence Alignments.

**(A)** Sequence alignment of Dem with YNV2 from yeast. Vertical lines show identical amino acids, and single and double dots represent similar amino acids, according to the Genetics Computer Group (Madison, WI) BESTFIT program.
**(B)** Amino acid sequence alignments of *Dem* with two Arabidopsis expressed sequence tags. F19919 is the 3′ end sequence, whereas N96644 is the 5′ end sequence.

The shoot apex of *dem* embryos lacks a shoot meristem and has a highly variable number of cotyledons that contain disorganized cells. During normal embryogenesis, two cotyledons are initiated on the apical flanks of a globular stage embryo, and the SAM becomes morphologically apparent between the emerging cotyledons (Jurgens et al., 1991; Mayer et al., 1991). In this context, we foresee a role for *Dem* in the organized cell divisions that accompany the transition between the globular and heart stages of embryogenesis. Without correct cell divisions, the normal signals that coordinate cotyledon number and apical development may be perturbed. This would lead to a deregulation of cotyledon number, altered cell division planes, and the failure to form or maintain a SAM. Because the true relationship between the cotyledons and the SAM in wild-type plants is not known (Kaplan, 1969; Barton and Poethig, 1993; Endrizzi et al., 1996), it is impossible to predict whether the SAM simply is not established in *dem* embryos or is established but not maintained.

In the *dem* root apex, cell divisions within a central zone of cells are disorganized, and several of the central cell files in the root are not correctly formed. *dem* roots terminate after a short period of growth but have the ability to initiate determinate lateral roots. The simplest explanation of the *dem* root phenotype is that the *dem* mutation makes roots determinate; primary roots and lateral roots can be formed, but they cannot be maintained. The fact that both terminal and lateral root meristems are determinate in *dem* mutants suggests that the *dem* SAM may also be determinate. These observations suggest that *Dem* may play a role in meristem maintenance and that the *dem* SAM is consumed in embryogenesis during the formation of cotyledons.

A possible clue for *Dem* function comes from the observation that two other mutants with altered cotyledon number have altered hormone levels. The Arabidopsis mutant *pinoid* (Bennett et al., 1995) frequently produces tricot seedlings and has defects in auxin transport. The mutant *altered meristem program1* also has a highly variable cotyledon number and altered cytokinin levels. Furthermore, polar auxin transport has been shown to be critical for pattern formation during embryogenesis (Liu et al., 1993), leaf and floral organ phyllotaxy (Meicenheimer, 1981; Okada et al., 1991), compound leaf development (Avasarala et al., 1996), meristem maintenance (Avasarala et al., 1996), and root meristem organization (Kerk and Feldman, 1995). Thus, *Dem* may be involved in cellular responses to hormone gradients that organize all apices and organ primordia.

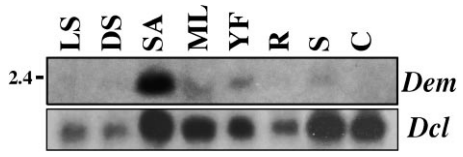


**Figure 7.** Tissue Distribution of *Dem* mRNA.

RNA gel blot analysis of *Dem* expression in light-grown seedlings (LS), dark-grown seedlings (DS), shoot apices (SA), mature leaves (ML), young fruit (YF), roots (R), stem (S), and callus (C). RNA was hybridized using the entire *Dem* cDNA, washed, and exposed for 48 hr. This blot was previously hybridized with a probe to *Dcl*, a constitutively and ubiquitously expressed mRNA (Keddie et al., 1996). A single message of 2.4 kb was observed.

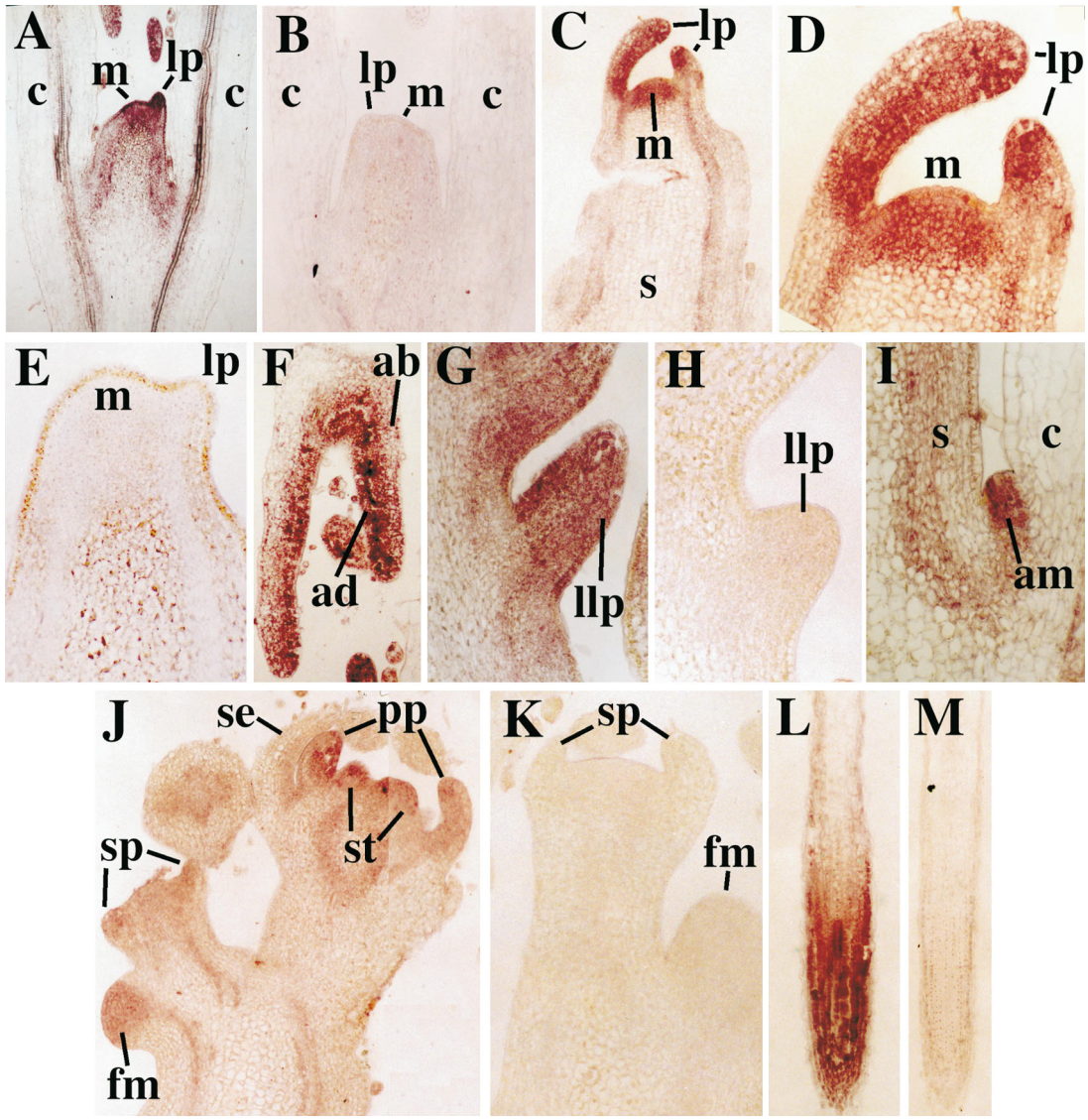


**Figure 8.** In Situ Distribution of *Dem* mRNA.

**(A)** and **(B)** SAM and cotyledons of 12-day-old seedlings. Signal was observed in the meristem and leaf primordia, using antisense **(A)** but not sense **(B)** *Dem* probes.
**(C)** to **(E)** SAM and stem from 4-week-old plants. Signal was observed in meristems and leaf primordia, using antisense **(C)** and **(D)** but not sense **(E)** probes.
**(F)** Cross-section showing *Dem* expression in the adaxial tissues of young leaves.
**(G)** and **(H)** *Dem* expression was detected in leaflet primordia, using sense **(G)** but not antisense **(H)** probes.
**(I)** Stem/cotyledon axis. *Dem* expression is detected in axillary meristems.
**(J)** and **(K)** Cross-section through inflorescence showing an emerging floral meristem and developing flowers. Staining is observed in floral meristems and in organ primordia as they emerge, using *Dem* antisense **(J)** but not *Dem* sense **(K)** probes.
**(L)** and **(M)** Sections through root tips. Staining was observed in root tips, using antisense **(L)** but not sense **(M)** probes.
Digoxigenin labeling is visible as brown staining. All hybridizations used 10-μm-thick sections and digoxigenin-labeled *Dem* probe. ab, abaxial; ad, adaxial; am, axillary meristem; c, cotyledon; fm, floral meristem; llp, leaflet primordium; lp, leaf primordia; m, meristem; pp, petal primordium; s, stem; se, sepal; sp, sepal primordium; st, stamen primordium.

A notable feature of the expression pattern of *Dem*, at least in shoot apices, is that it is apparently coincident with the expression of *tKn1*, a gene encoding a KNOTTED1-related homeodomain protein of tomato (Hareven et al., 1996). KNOTTED1-related proteins are believed to maintain cells in an undifferentiated state within meristems (Smith et al., 1992) and in the leaf and leaflet primordia of tomato (Hareven et al., 1996). Similar to *Dem*, *knotted1*-related genes of maize are not expressed in callus tissue (Smith et al., 1992) and are expressed in vascular strands (Smith et al., 1992; Jackson et al., 1994). Also, in Arabidopsis, mutations in *STM*, a *Knotted1* homolog, result in seedlings with no apparent SAM (Long et al., 1996). These observations suggest that *Dem* may be required for correct cell division patterns within the domain of *Knotted* expression.

In summary, we have identified a mutant, *dem*, that plays an important role in the maintenance or function of both the SAM and RAM. We have cloned the *Dem* gene by transposon tagging and shown that it is expressed in all areas of the plant in which organized cell division is taking place. The conceptual translation of the *Dem* cDNA provides little evidence regarding the function of the Dem protein. The lack of apparent nuclear localization sequences or DNA binding motifs suggests that it is not a nuclear transcription factor. The presence of myristoylation consensus motifs makes it tempting to speculate that Dem may be anchored to a cellular membrane. The homology of Dem to a yeast protein raises the possibility that Dem is a cellular component that has evolved to become an essential gene for organized cell divisions that occur in meristems and primordia during plant development.

## METHODS

### Transgenic Plant Material and Generation of the *defective embryo and meristems* Mutant

Transgenic tomato (*Lycopersicon esculentum*) cultivar Moneymaker carrying maize transposable elements was used for all experiments. A total of 150 transposants was generated from a single *Dissociation* (*Ds*) T-DNA line (1561E) by selection for excision and reinsertion of *Ds* after testcrossing a 1561E/10512I double heterozygote to wild-type plants (Carroll et al., 1995). The 10512I line carries the transposase gene (*sAc*) linked to β-glucuronidase (GUS). Seedlings carrying a transposed *Ds* were self-pollinated, and the progeny were screened for mutations. Family N174 carries a single transposed *Ds* and includes mutants exhibiting the *defective embryo and meristems* (*dem*) phenotype.

### Reversion of the *dem* Mutant in the Presence of the Transposase

To demonstrate instability of *dem* in the presence of a transposase, a *Dem* heterozygote was crossed to the transposase line 10512I (Carroll et al., 1995). $F_1$ double heterozygotes for the *Ds* insertion and the transposase gene were identified by a polymerase chain reaction (PCR) test (identifying $dem^{Ds}$; see below) and histochemical staining for GUS (the marker for the transposase gene). $F_1$ double heterozygotes were selfed, and the $F_2$ generation was screened for GUS-positive mutant seedlings. GUS-positive mutants were observed for somatic instability of the mutant phenotype. Somatic $F_2$ revertants were testcrossed to an untransformed tester, and the progeny were screened for germinal wild-type and mutant excision alleles at the *Dem* locus, as described below.

### Cloning the *Dem* cDNA

Fragments of the *Dem* gene were cloned by inverse PCR (IPCR) (Thomas et al., 1994) and used to screen a λgt10 cDNA library constructed using seedling mRNA. We purified six positives from $5 \times 10^5$ plaques, and one full-length *Dem* cDNA was sequenced on both strands. RNA and genomic DNA extraction and analysis were performed as described previously (Keddie et al., 1996).

### PCR Test for *Ds* Zygosity at the *Dem* Locus

The mutant line was maintained as a heterozygote. To detect zygosity for the *Ds* insertion in *Dem*, we developed a simple triplex PCR test (Thomas et al., 1994) with intact leaf tissue (Klimyuk et al., 1993; Carroll et al., 1995). Based on the sequences flanking both sides of the *Ds* in *dem*, oligonucleotide primers dem5′ (5′-TTTCTGCTCCTTAAATGCATTGAG-3′) and dem3′ (5′-TTCATGTTGGTGGGAACACTGCGA-3′) were designed to amplify a 220-bp preinsertion fragment. dem5′, in combination with primer B34 (5′-ACGGTCGGTACGGGATTTTCCCAT-3′), which primes from sequences at the end of *Ds*, amplifies a 154-bp fragment corresponding to the *Ds* insertion in the *dem* gene. By using PCR with these three primers, we performed zygosity tests for the *Ds* insertion in *Dem* on individual seedlings.

### PCR Footprint Analysis of *Dem* Revertants

Footprint analysis was done using oligonucleotides dem5′ and dem3′. PCR products from wild-type and germinal revertant plants were cloned and sequenced. In addition, a screen for new excision alleles was performed by crossing $sAc^+$ $dem^{+7}$ heterozygotes with $dem^{Ds}$ heterozygotes. The seeds from this cross were germinated, and wild-type plants were discarded. After ~1 month, ~50% of the mutants initiated a shoot from between their cotyledons, and growth was resumed. To analyze the size of footprints left in new *dem* alleles, either dem3′ or dem5′ was kinase labeled with γ-labeled $^{33}$P-ATP and used with the other primer to amplify excision alleles. PCR products were denatured and separated on a 6% polyacrylamide gel (Figure 4B).

### Microscopy and in Situ Hybridization

Samples for light microscopy were prepared using a microwave procedure. Tissue was fixed twice in formaldehyde acetic acid (FAA) at 37°C for a total of 30 min, dehydrated at 67°C in 70% ethanol and then 100% ethanol for 75 sec each, treated in 2-propanol at 75°C for 90 sec, and then embedded in molten Paraplast (Pelco, Reading, CA) at 67°C for ~3 hr in a 3440 MAX Laboratory microwave (Pelco). A full version of this protocol can be obtained from the National Science

Foundation Center home page (www.plantbio.berkeley.edu). Samples were serially sectioned, stained in safranin O and orange gold to highlight densely cytoplasmic cells, and viewed on an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). Samples prepared for scanning electron microscopy (SEM) were fixed in FAA, dehydrated in ethanol, dried in a critical point dryer, sputter coated with palladium to 20 nm, and viewed on a DS130 scanning electron microscope (ISI, Philadelphia, PA).

In situ RNA hybridization was performed using methods described by Coen et al. (1990). An internal 559-bp EcoRI fragment of the *Dem* cDNA was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA). T7- and T3-primed digoxigenin-labeled RNA probes were made using digoxigenin RNA labeling mix (Boehringer Mannheim) and hydrolyzed at 60°C for 30 min in 100 mM carbonate buffer, pH 10.2. A minimum of three samples were examined per experiment, and sense strand controls were always included.

## ACKNOWLEDGMENTS

We thank the staff of the University of California at Berkeley National Science Foundation Center for Plant Developmental Biology for training, colleagues at the University of California at Berkeley for stimulating discussion about meristems, and members of the Gruissem laboratory for critical reading of the manuscript and advice. J.S.K. was supported by a Human Frontiers Science Program Long-Term Fellowship. B.J.C. is grateful to the University of Queensland for an Australian Research Council grant. Research in the laboratory of W.G. is supported by the National Science Foundation, and research in the Sainsbury Laboratory is funded by the Gatsby Charitable Trust.

Received January 13, 1998; accepted March 20, 1998.

## REFERENCES

**Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J.** (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. **25,** 3389–3402.

**Avasarala, S., Yang, J., and Caruso, J.L.** (1996). Production of phenocopies of the *Lanceolate* mutant in tomato using auxin transport inhibitors. J. Exp. Bot. **47,** 709–712.

**Ball, E.** (1952). Morphogenesis of shoots after isolation of the shoot apex of *Lupinus albus.* Am. J. Bot. **39,** 167–191.

**Barton, M.K., and Poethig, R.S.** (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana*: An analysis of development in the wild-type and in the *SHOOT MERISTEMLESS* mutant. Development **119,** 823–831.

**Benfey, P.N., and Schiefelbein, J.W.** (1994). Getting to the root of plant development: The genetics of *Arabidopsis* root formation. Trends Genet. **10,** 84–88.

**Bennett, S.R.M., Alvarez, J., Bossinger, G., and Smyth, D.R.** (1995). Morphogenesis in *pinoid* mutants of *Arabidopsis.* Plant J. **8,** 841–857.

**Carroll, B.J., Klimyuk, V.I., Thomas, C.M., Bishop, G.J., Harrison, K., Scofield, S., and Jones, J.D.G.** (1995). Germinal transpositions of the maize element *Dissociation* from T-DNA loci in tomato. Genetics **139,** 407–420.

**Caruso, J.L.** (1968). Morphogenetic aspects of a leafless mutant in tomato. I. General patterns of development. Am. J. Bot. **55,** 1169–1176.

**Clark, S.E., Williams, R.W., and Meyerowitz, E.M.** (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell **89,** 575–585.

**Coen, E.S., Romero, J.M., Doyle, S., Elliot, R., Murphy, G., and Carpenter, R.** (1990). Floricaula: A homeotic gene required for flower development in *Antirrhinum majus.* Cell **63,** 1311–1322.

**Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B.** (1993). Cellular organization of the *Arabidopsis thaliana* root. Development **119,** 71–84.

**Endrizzi, K., Moussian, B., Haecker, A., Levin, J.Z., and Laux, T.** (1996). The *SHOOT MERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes *WUSCHEL* and *ZWILLE.* Plant J. **10,** 967–979.

**Feldman, L.J., and Torrey, J.G.** (1976). The isolation and culture *in vitro* of the quiescent center of *Zea mays.* Am. J. Bot. **52,** 580–590.

**Hareven, D., Gutfinger, T., Parnis, A., Eshed, Y., and Lifschitz, E.** (1996). The making of a compound leaf: Genetic manipulation of leaf architecture. Cell **84,** 735–744.

**Jackson, D., Veit, B., and Hake, S.** (1994). Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development **120,** 405–413.

**Jurgens, G., Mayer, U., Torres-Ruiz, R.A., Berleth, T., and Misera, S.** (1991). Genetic analysis of pattern formation in the *Arabidopsis* embryo. Development **113** (suppl.), 27–38.

**Kaplan, D.** (1969). Seed development in *Downingia.* Phytomorphology **19,** 153–178.

**Keddie, J.S., Carroll, B., Jones, J.D.G., and Gruissem, W.** (1996). The *DCL* gene of tomato is required for chloroplast development and palisade cell morphogenesis in leaves. EMBO J. **15,** 4208–4217.

**Kerk, N.M., and Feldman, J.L.** (1995). A biochemical model for the initiation and maintenance of the quiescent center: Implications for organization of root meristems. Development **121,** 2825–2883.

**Klimyuk, V., Carroll, B.J., Thomas, C.M., and Jones, J.D.G.** (1993). Alkali treatment for rapid preparation of plant tissue for reliable PCR analysis. Plant J. **3,** 493–494.

**Liu, C.-m., Xu, Z.-h., and Chua, N.-H.** (1993). Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell **5,** 621–630.

**Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K.** (1996). A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis.* Nature **353,** 402–407.

**Lucas, W.J., Bouche-Pillon, S., Jackson, D., Nguyen, L., Baker, L., Ding, B., and Hake, S.** (1995). Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Science **270,** 1980–1983.

**Lyndon, R.F.** (1990). Plant development: The cellular basis. In Topics in Plant Physiology, M. Black and J. Chapman, eds (London: Unwin Hyman), pp. 181–238.

**Mayer, U., Torres-Ruiz, R.A., Berleth, T., Misera, S., and Jurgens, G.** (1991). Mutations affecting body organization in the *Arabidopsis* embryo. Nature **353,** 402–407.

**McCully, M.E.** (1975). The development of lateral roots. In The Development and Function of Roots, J.G. Torrey and D.T. Clarkson, eds (New York: Academic Press), pp. 39–58.

**Meicenheimer, R.D.** (1981). Changes in *Epilobium* phyllotaxy induced by *N*-1-naphthylphthalamic acid and $\alpha$-4-chlorophenoxyisobutyric acid. Am. J. Bot. **68,** 1139–1154.

**Meyerowitz, E.M.** (1997). Genetic control of cell division patterns in developing plants. Cell **88,** 299–308.

**Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y.** (1991). Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. Plant Cell **3,** 677–684.

**Poethig, R.S.** (1987). Clonal analysis of cell lineage patterns in plant development. Am. J. Bot. **74,** 581–594.

**Saedler, H., and Nevers, P.** (1985). Transposition in plants: A molecular model. EMBO J. **4,** 585–590.

**Smith, L., Greene, B., Veit, B., and Hake, S.** (1992). A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. Development **116,** 21–30.

**Steeves, T.A., and Sussex, I.M.** (1991). Patterns in Plant Development. (Cambridge, UK: Cambridge University Press).

**Thomas, C.M., Jones, D.A., English, J., Carroll, B.J., Bennetzen, J.L., Harrison, K., Burbridge, A., Bishop, G.J., and Jones, J.D.G.** (1994). Analysis of the chromosomal distribution of transposase-carrying T-DNAs in tomato using the inverse polymerase chain reaction. Mol. Gen. Genet. **242,** 573–585.