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Role of *WUSCHEL* in Regulating Stem Cell Fate in the *Arabidopsis* Shoot Meristem

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Summary

The shoot meristem gives rise to the aerial parts of higher plants by continuously initiating new organs. The basis of this activity is its ability to maintain a pool of pluripotent stem cells, which are the ultimate source of all tissues of the shoot. In *Arabidopsis* plants mutant for the *WUSCHEL* (*WUS*) gene, the stem cells are misspecified and appear to undergo differentiation. Here, we show that *WUS* encodes a novel homeodomain protein which presumably acts as a transcriptional regulator. The pattern of *WUS* expression suggests that stem cells in the shoot meristem are specified by an underlying cell group which is established in the 16-cell embryo and becomes localized to its prospective domain of function by asymmetric cell divisions.

Introduction

Higher plants form organs throughout their life via the activity of apical meristems (Steeves and Sussex, 1989). During plant embryogenesis only a basic body organization is laid down with a root and a shoot meristem at the basal and apical pole of the seedling, respectively. The meristems contain a pool of pluripotent stem cells, which are able to self-maintain and to produce the cells required for organ initiation. The initiation and maintenance of stem cells and their integration into organ-forming meristems are thus the basis for continuous postembryonic plant development.

The origin and development of the shoot meristem in the embryo have been discussed controversially. Based on comparative morphology, one model in classical botany holds that the shoot meristem is first differentiated by the simultaneous initiation of the cotyledons and a perpetuating center in the apical region of the globular embryo (Spurr, 1949; Kaplan, 1969; Kaplan and Cooke, 1997). Alternatively, the shoot meristem has been interpreted as the result of an embryonic patterning process (Hake et al., 1995; Laux and Jürgens, 1997; Long and Barton, 1998; Moussian et al., 1998). Histologically, the shoot meristem can be recognized at mid-embryo stages (Barton and Poethig, 1993). The expression of the shoot meristem-specific *SHOOTMERISTEMLESS* (*STM*) gene is observed earlier in cells between the incipient cotyledonary primordia of the globular-stage embryo (Long et al., 1996). However, nothing is known about the origin of the shoot meristem and its development before this stage.

In postembryonic development, the *Arabidopsis* shoot meristem first gives rise to a rosette of leaves with axillary meristems and later to floral meristems. Floral meristems are homologous to shoot meristems, and their activity is regulated in part by the same set of genes (Clark et al., 1993; Endrizzi et al., 1996; Laux et al., 1996). However, in contrast to *Arabidopsis* shoot meristems, floral meristems give rise to a fixed number of organs and terminate in the central gynoecium.

In the shoot meristem, a central zone (CZ) at the summit where cells divide relatively infrequently can be distinguished from the surrounding peripheral zone (PZ) with rapidly dividing cells (Figure 1A; Steeves and Sussex, 1989). The CZ is thought to harbor the stem cells, whereas organ formation is initiated in the PZ (Steeves and Sussex, 1989). From the outside to the inside, the shoot meristem is organized into three separate "germ" layers that contribute differentially to the tissues of the shoot (Figure 1A; Satina et al., 1940). Cells in the outermost layer, L1, give rise to the epidermis, whereas derivatives of the two subepidermal layers, L2 and L3, form internal tissues. Clonal analysis indicated that each cell layer contains 1–3 stem cells, from which all cells within this layer are derived (Stewart and Dermen, 1970).

To maintain the integrity of the shoot meristem during repetitive organ formation, cell proliferation and organ initiation must be precisely coordinated (Clark, 1997; Meyerowitz, 1997). The balance between these two processes appears to be competitively regulated by the *CLAVATA* (*CLV*) and *STM* genes (Clark et al., 1996). CLV1, a putative receptor kinase, is likely to be a component of a signaling pathway that promotes organ formation (Clark et al., 1997; Williams et al., 1997; Laufs et al., 1998). Its antagonist, STM, is a homeodomain protein of the KNOTTED-1 family (Kerstetter et al., 1994; Long et al., 1996) and appears to prevent the entry of cells into organogenesis (Clark et al., 1996; Endrizzi et al., 1996).

Little is known, however, about the mechanisms by which the stem cells are specified during the formation and maintenance of shoot and floral meristems. The stem cells are pluripotent in that they give rise to a variety of cell types, and they are undifferentiated in that they lack features of cells in mature tissue (Barlow, 1978; Steeves and Sussex, 1989). Clonal analysis (Ruth et al., 1985) indicated that stem cell fate is specified by positional information and imposed on the cells that currently reside at the summit of the shoot meristem (reviewed in Newman, 1965; Laux and Mayer, 1998). Only the progeny of stem cells that stay at this position remain pluripotent, whereas daughter cells that leave this position differentiate.

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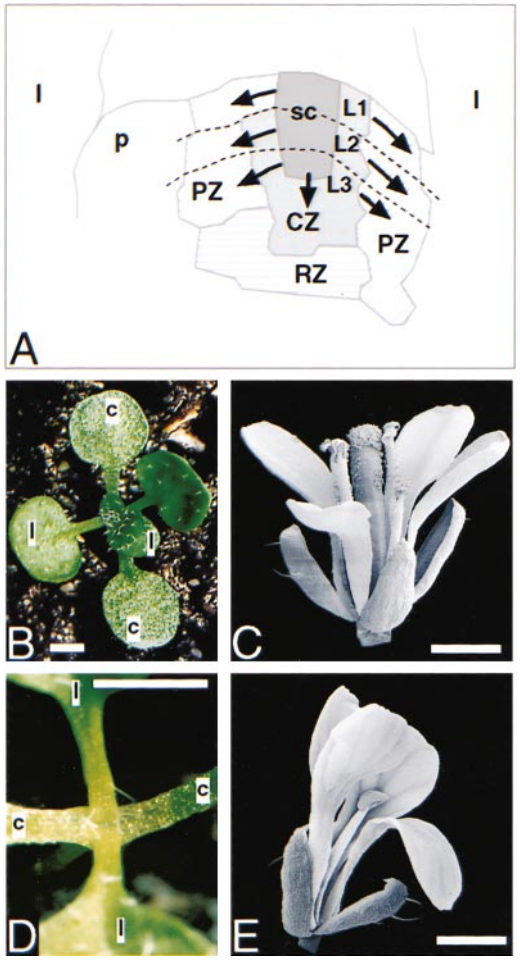


Figure 1. Shoot and Floral Meristem Development

(A) Organization of the shoot meristem. The tissues of the plant shoot are derived from three germ layers of the shoot meristem, L1–L3 (arrows). Each layer presumably contains 1–3 stem cells (sc, darkly shaded) within the apical part of the central zone (CZ, faintly shaded). Organ primordia (p) are initiated in the peripheral zone (PZ, hatched). Underneath the CZ, the flat cells of the rib zone (RZ, horizontal stripes) form the central pith of the shoot axis.

(B and D) Live seedlings. (B) Wild-type seedlings give rise to a rosette of leaves. (D) *wus* seedling apices discontinue leaf formation after two true leaves have been formed. c, cotyledon; I, leaf.

(C and E) Scanning electron micrographs of flowers. (C) Wild-type flowers contain four whorls of organs: four sepals in the first, four petals in the second, six stamens in the third, and two fused carpels in the fourth whorl. (E) *wus* flowers, which are occasionally formed on adventitious shoots, display normal organ numbers in the two outer whorls but terminate in a single central stamen.

Scale bars, 1 mm (B and D) and 500 μm (C and E).

Recent genetic analysis in *Arabidopsis* suggests a central role for the *WUS* gene in regulating stem cell fate throughout development. Mutations in the *WUS* gene specifically result in the failure of shoot and floral meristems to self-maintain (Figures 1B–1E) (Laux et al., 1996). Although in the absence of WUS, stem cells appear misspecified, they are not incorporated into organs, indicating that *WUS* is required to specify stem cell fate rather than to repress organ formation. Genetic analysis indicated that *WUS* interacts with several other regulators of shoot and floral meristems, suggesting that *WUS* integrates positional and temporal information from these genes (Laux et al., 1996; Laux and Schoof, 1997).

Here, we report the molecular analysis of the *WUS* gene, addressing its role in shoot and floral meristems. Our data support a novel model to explain how these meristems are formed and maintained in plant development. We provide evidence that the specification of stem cells requires a small group of underlying cells that exhibit *WUS* expression. This cell group originates during early embryo pattern formation long before a shoot meristem can be recognized.

Results

Map-Based Cloning of the *WUS* Gene

To isolate the *WUS* gene, we used RFLP- and PCR-based mapping of recombination breakpoints in 3150 meiotic events from a cross of *wus-1* (L*er*) × wild type (Nd). We determined the order of genomic loci as ABI14-GII, pCITd84, m216, *WUS*, and PhyB and localized *WUS* about 0.8 cM proximal to the RFLP marker pCITd84 (Figure 2A). We initiated a chromosome walk from pCITd84 with yeast artificial chromosomes (YAC) and localized *WUS* on a single YAC clone of about 400 kb, yUP3C8. From the right end of this YAC, we initiated a cosmid walk spanning about 52 kb and mapped the *WUS* gene to this contig (Figure 2A).

To further localize the *WUS* gene, we transformed cosmids representing this contig into roots of homozygous *wus-1* seedlings and examined the regenerated transgenic plants for complementation. The seedlings had been obtained from a cross of *wus-1* (L*er*) × wild type (Ws-2), which enabled us to confirm their genotype at the *WUS* locus by PCR for the closely linked markers m216 and yUP3C8 right (Figure 2A). Roots taken from 14 seedlings that were transformed with cosmids 6, 8, or 9 (Figure 2A) gave rise to a total of 76 transgenic plants that showed complementation of the *wus* defect and formed flowers with a complete set of organs (Figure 2B). By contrast, all transgenic plants containing any of the other cosmids showed the *wus* mutant phenotype. The progeny of the plants that showed complementation segregated *WUS* and *wus* phenotypes. All of 338 kanamycin-resistant plants tested exhibited a wild-type phenotype, indicating that the T-DNA that conferred kanamycin resistance also carried the *WUS* gene (data not shown).

The three complementing cosmids shared two HindIII DNA fragments of 10 and 1.5 kb, respectively. With these fragments, we screened 5×10^5 clones of a cDNA library made from young flowers (Weigel et al., 1992) and isolated seven independent cDNA clones, which represented a single transcript. Two clones displayed one long open reading frame of 873 nucleotides. We confirmed that this open reading frame represented the *WUS* gene by identifying mutations in the cognate genomic DNA of four different *wus* alleles (see below). Stop codons upstream of this open reading frame indicated that these cDNAs contained the complete coding region.

The *WUS* Gene Encodes a Homeodomain Protein

The putative transcription start of the *WUS* gene was determined by 5′-RACE. We analyzed the genomic sequence upstream of the *WUS* gene and identified a TATA box, a CCAAT box, and a GC box at 35 bp, 56 bp, and 80 bp upstream of the transcription start, respectively (data not shown). A putative polyadenylation signal (Wu et al., 1995) was identified 20 bp upstream of the poly(A) tail (data not shown). A comparison of genomic and cDNA sequences revealed the presence of two introns with a length of 566 bp and 90 bp, respectively (Figure 2C). The *WUS* gene encodes a conceptual protein of 291 amino acids, and sequence comparison revealed two putative functional domains (Figure 2C). The region between amino acid residues 33–98 shows similarity to homeodomains (see below), and the region between residues 234–241 contains a cluster of acidic amino acid residues. Computer analysis predicted that the acidic cluster can form an amphipathic α helix, similar to known transactivation domains of transcriptional regulators (Ptashne, 1988).

WUS Represents a Novel Subtype of the Homeodomain Protein Family

Homeodomain proteins have been identified in many organisms and generally appear to be involved in developmental or cell type regulation. The characteristic homeodomain is a conserved protein domain of about 60 amino acids that functions in sequence-specific DNA binding. The hallmarks are a helix-loop-helix-turn-helix structure and 12 highly conserved amino acid residues (Gehring et al., 1990; Laughon, 1991). The WUS homeodomain has all of these properties and was found to be about 30% identical to and 45%–50% similar to several homeodomain sequences from plants, animals, and yeast (Figure 2D). To determine the relationship of WUS to other homeodomain proteins, we performed a similarity analysis using a heuristic tree-building program. This analysis revealed that the WUS homeodomain cannot be significantly grouped together with any other homeodomain (data not shown) and therefore represents a novel branch on the homeodomain protein tree.

Mutations Disrupting the *WUS* Gene

Based on the average number of central stamens formed in mutant flowers, we classified the three mutant alleles *wus-1*, *wus-2*, and *wus-4* as strong (about one stamen per flower) and *wus-3* as a weak allele (about three stamens per flower). The *WUS* gene was amplified and sequenced from genomic DNA of homozygous *wus* mutants. In each allele a single point mutation relative to wild type was detected (Figure 2C). The mutations in the three strong *wus* alleles presumably lead to a premature translational stop. In *wus-4*, a TAA stop codon results in a translational stop after 44 amino acid residues. In *wus-1*, the 5′-exon-intron boundary of intron 2 is changed from GG to GA, replacing a G that is highly conserved at plant gene splice sites (Brown, 1996). The failure to remove intron 2 results in a translational stop after a few codons within the intron (data not shown). *wus-2* contains a TAG stop codon at amino acid position 209. In *wus-1* and *wus-2*, a translational stop results in the loss of about one-third of the protein, including the putative transactivation domain. The weak *wus-3* allele contains a proline to leucine missense mutation in the N-terminal part of the homeodomain.

Cellular Localization of the WUS Protein

In order to study the subcellular localization of the WUS protein, we used a translational fusion between *WUS* and the Glucuronidase (GUS) reporter gene in transient expression assays (Varagona et al., 1992). Figure 3 shows that WUS targeted most GUS activity into the nucleus of onion epidermis cells. The nuclear localization activity of WUS is consistent with its proposed role as a transcriptional regulator.

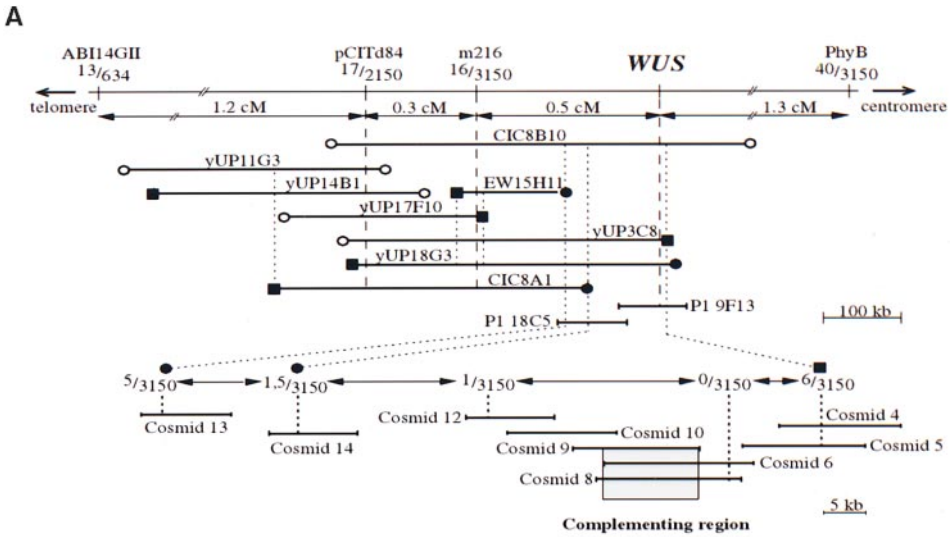
WUS Expression during Embryogenesis

Phenotypic analyses had suggested that *WUS* is required for pluripotent cell fate during embryonic shoot meristem development (Laux et al., 1996). To test this hypothesis, we analyzed *WUS* mRNA expression during embryogenesis. Due to an invariant cell division pattern in initial stages of *Arabidopsis* embryo development, morphological regions of the seedling can be traced back to cells in the early embryo (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). The eight-cell embryo consists of an apical region of four cells, which will form the shoot meristem and most of the cotyledons, and a basal region of four cells, which will form the hypocotyl and most of the root. Up to the eight-cell embryo stage, no *WUS* expression was detected (Figure 4A).

Each of the eight embryo cells divides periclinally to give an inner cell and an outer protoderm cell. This was the earliest stage at which *WUS* mRNA was detected. It was confined to the four inner cells of the apical region (Figure 4B). Each of these inner cells then divides longitudinally, giving rise to a central and a peripheral daughter. *WUS* mRNA was only detected in central daughter cells but was absent from peripheral daughters (Figures 4C and 4D).

The transition-stage embryo consists of about 100 cells and is characterized by the outgrowth of cotyledonary primordia on each side of the presumptive shoot meristem region (Figure 4E). At this stage, *WUS* mRNA was detected in two subepidermal cells in the center of the apex (Figure 4E). Serial sections suggested that the *WUS* expression domain was only one cell deep (data not shown).

At the heart stage, the basic body organization, comprising cotyledonary primordia, shoot meristem primordium, hypocotyl primordium, and root pole, is histologically recognizable. The subepidermal cells within the shoot meristem region have divided transversely, giving rise to upper (L2) and lower (L3) daughter cells. *WUS* mRNA was restricted to the L3 daughter cells but was absent from the L2 daughters (Figure 4F). Longitudinal sections perpendicular to the plane of the cotyledons revealed that the *WUS* expression domain spans only one cell in depth (Figure 4G). Until this stage, we have not found a single case ($n = 60$) in which after a cell division both daughter cells contained *WUS* mRNA, indicating that asymmetric *WUS* mRNA distribution was established during or rapidly after cell division.



C

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M E P P Q H Q H H H   H Q A D Q E S G N N   N N K S G S G G Y T    30
C R Q T S T R W T P   T T E Q I K I L K E   L Y Y N N A I R S P    60
T A D Q I Q K I T A   R L R Q F G K I E G   K N V F Y W F Q N H    90
K A R E R Q K K R F   N G T N M T T P S S   S P N S V M M A A N   120
D H Y H P L L H H H   H G V P M Q R P A N   S V N V K L N Q D H   150
H L Y H H N K P Y P   S F N N G N L N H A   S S G T E C G V V N   180
A S N G Y M S S H V   Y G S M E Q D C S M   N Y N N V G G G W A   210
N M D H H Y S S A P   Y N F F D R A K P L   F G L E G H Q D E E   240
E C G G D A Y L E H   R R T L P L F P M H   G E D H I N G G S G   270
A I W K Y G Q S E V   R P C A S L E L R L   N                   291
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L (*wus-3*) *(*wus-4*) *(*wus-1*) *(*wus-2*)

D

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                  helix I      loop      helix II      turn      helix III
WUS       QTSTRWTPTTEQIKILKELYYNNAIRSPTADQIQKITARLRQFGKIEGKNVFYWFQNHKARERQKK
AtHB8,A   GKYVRYTP--EQVEALERLYNDC--PKPSSMRRQQLIRECPILSNIEPKQIKVWFQNRRCREKQRK
AtHB14,A  GKYVRYTP--EQVEALERVYTEC--PKPSSLRRQQLIRECPILSNIEPKQIKVWFQNRRCREKQRK
AtHB9,A   GKYVRYTP--EQVEALERVYAEC--PKPSSLRRQQLIRECPILCNIEPRQIKVWFQNRRCREKQRK
ATBF1,H   DKRLRTTITPEQLEILYQKYLLD--SNPTRKMLDHIAHEV----GLKKRVVQVWFQNTRARERKGQ
SHOXB,H   QRRSRTNFTLEQLNELERLFDET--HYPDAFMREELSQRL----GLSEARVQVWFQNRRAKCRKQE
en,B      EKRPRTAFTSEQLQRLKKEFQEN--RYLTEQRRQDLAREL----KLNEsQIKIWFQNKRAKIKKAT
LIM3,X    AKRPRTTITAKQLETLKNAYNNS--PKPARHVREQLSSET----GLDMRVVQVWFQNRRAKEKRLK
Pho2,S    QRPKRTRAKGEALDVLKRKFEIN--PTPSLVERKKISDLI----GMPEKNVRIWFQNRRAKLRKKQ
ZFH1,D    KVRVRTAINEEQQQQLKQHYSLN--ARPSRDEFRMIAARL----QLDPRVVQVWFQNNRSRERKMQ
HOXA13,H  GRKKRVPYTKVQLKELEREYATN--KFITKDKRRRISATT----NLSERQVTIWFQNRRVKEKKVI
ZFH2,D    QKRARTRITDDQLKILRAHFDIN--NSPSEESIMEMSQKA----NLPMKVVKHWFRNTLFKERQRN
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Figure 2. Positional Cloning and Structure of the *WUS* Gene

(A) A 1.0 cM region between the RFLP marker locus pCitd84 and the right end of yUP3C8 was spanned with YAC, P1, and cosmid clones. Cosmids 4–6, 8–10, and 12 were tested for complementation of the *wus-1* mutation. The complementing region is indicated by shading. The frequency of recombinants found with a given marker is indicated.

(B) Comparison of a flower from a *wus-1* plant (*wus*) that terminates in a single stamen and does not form a gynoecium and a flower from a *wus-1* plant transformed with cosmid 9 (rescue), which formed a gynoecium and was indistinguishable from wild-type flowers (compare with Figure 1C).

(C) The WUS protein sequence deduced from the longest open reading frame of the *WUS* cDNA. The mutations in *wus-2* and *wus-4* result in

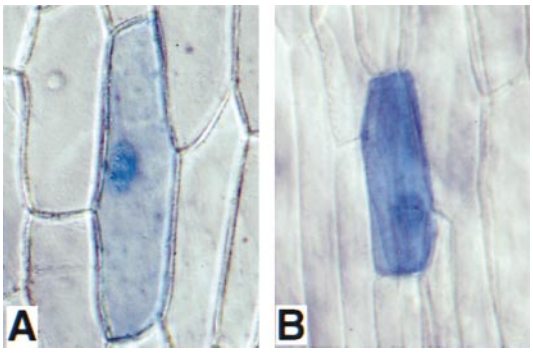

Figure 3. Nuclear Localization of the WUS-GUS Protein

(A) WUS-GUS fusion protein is localized in the nucleus of onion epidermis cells.

(B) Negative control: GUS protein is evenly distributed throughout the cell.

During subsequent development, the embryonic shoot meristem increases in size and initiates the first two leaf primordia. In late-stage embryos, *WUS* mRNA was detected in a small group of central cells in and underneath the L3 of the shoot meristem (Figure 4H). By contrast, *WUS* mRNA was absent from the two outer cell layers. In summary, *WUS* expression was initiated in the 16-cell embryo and was gradually confined to the center of the developing embryonic shoot meristem, but it was absent from all other parts of the embryo.

WUS and *STM* Gene Expression Are Initiated Independently of Each Other

In *stm* mutants the shoot meristem terminates prematurely in fused organs. Genetic analysis showed that mutations in the *WUS* gene did not lead to morphological changes in seedlings of strong *stm* alleles (Endrizzi et al., 1996). However, *WUS* expression is initiated in shoot meristem precursor cells several embryo stages before *STM* is expressed. To examine the relation between *WUS* and *STM* at the transcriptional level, we used in situ hybridization in *wus* and *stm* late-stage embryos, in which the mutant phenotypes could be scored unambiguously. In late-stage *stm* embryos, there is no wild-type shoot meristem, and the cotyledonary primordia join at an acute angle (Figure 4K). At this stage, we observed strong *WUS* expression in apical cells corresponding to the position of the shoot meristem in wild type, indicating that there are at least some cells in the *stm* apex that have meristematic identity. We were, however, unable to detect *WUS* expression in apices of *stm* seedlings (data not shown). The *STM* gene is expressed throughout the shoot meristem in late-stage wild-type embryos (Figure 4L). In *wus* there are a few slightly enlarged cells in place of the shoot meristem at this stage (Laux et al., 1996). At least some of these cells expressed *STM* (Figure 4M). However, we did not detect *STM* expression in terminated apices of *wus* seedlings (data not shown). Therefore, expression of both *WUS* and *STM* during embryo development appears to be initiated independently of the respective other gene, but it was no longer found in apparently differentiated apices of the corresponding mutant seedlings.

WUS Expression in Postembryonic Development

The *wus* phenotype suggested that during postembryonic development, *WUS* is required in central cells of shoot and floral meristems (Laux et al., 1996). To test this hypothesis, we studied *WUS* expression in postembryonic shoot meristems and during flower development. In both the vegetative (Figure 5A) and the inflorescence shoot meristem (Figure 5B), *WUS* mRNA was confined to a small group of cells in the center of the shoot meristem underneath the three outermost cell layers.

Floral meristems emerge as small bulges (stage 1 in Figure 5B; stages according to Bowman, 1994) at the periphery of the inflorescence meristem and develop into a dome of cells (stage 2, Figure 5D). Subsequently, sepals, petals, and stamens are successively initiated at the periphery of the floral meristem (Figures 5B and 5E). The floral meristem terminates when its central cells are consumed during the initiation of carpel primordia (Figure 5F). *WUS* mRNA was detected in stage 1 floral meristems in a region separate from the *WUS* expression domain of the inflorescence meristem, suggesting that *WUS* expression was newly established during flower development (Figures 5B and 5C). Subsequently, the strongest *WUS* expression was observed in stage 2 flowers (Figures 5C and 5D), after which the hybridization signal decreased (Figure 5E) to become undetectable when the carpel primordia emerged (Figure 5F). In contrast to the shoot meristem, floral meristems accumulated *WUS* mRNA in a small group of central cells that included the third cell layer. *WUS* mRNA was only excluded from the two outermost layers.

Discussion

We have reported the cloning of the *WUS* gene, a central regulator of stem cell fate in shoot and floral meristems, and have addressed its role during embryonic and postembryonic meristem development.

stop codons (designated *). The mutation in *wus-1* changes an exon–intron border and results in a predicted translational stop a few codons later (designated *). The mutation in *wus-3* results in an amino acid substitution as indicated. The homeodomain (solid line) and an acidic domain (broken line) are underlined. The positions of introns are indicated by triangles.

(D) Comparison of the WUS protein region between residues 33 and 98 with homeodomain sequences identified in the GenBank database. Residues identical or similar (Gribskov et al., 1986) to the WUS sequence are shaded. The highly conserved residues in homeodomains are indicated (*) (Gehring et al., 1990). Gaps are given as dashes. GenBank accession numbers: LIM3, Z22702; ZFH1, M63449; ATBF1, D10250; Pho2, M24613; ZFH2, M63450; SHOXB, U82668; HOXA13, U82827; engrailed (en), U82487; AtHB8, Z50851; AtHB9, Y10922; AtHB14, Y11122. A, *Arabidopsis thaliana*; B, *Branchiostoma floridae*; D, *Drosophila melanogaster*; H, human; S, *Saccharomyces cerevisiae*; and X, *Xenopus*.

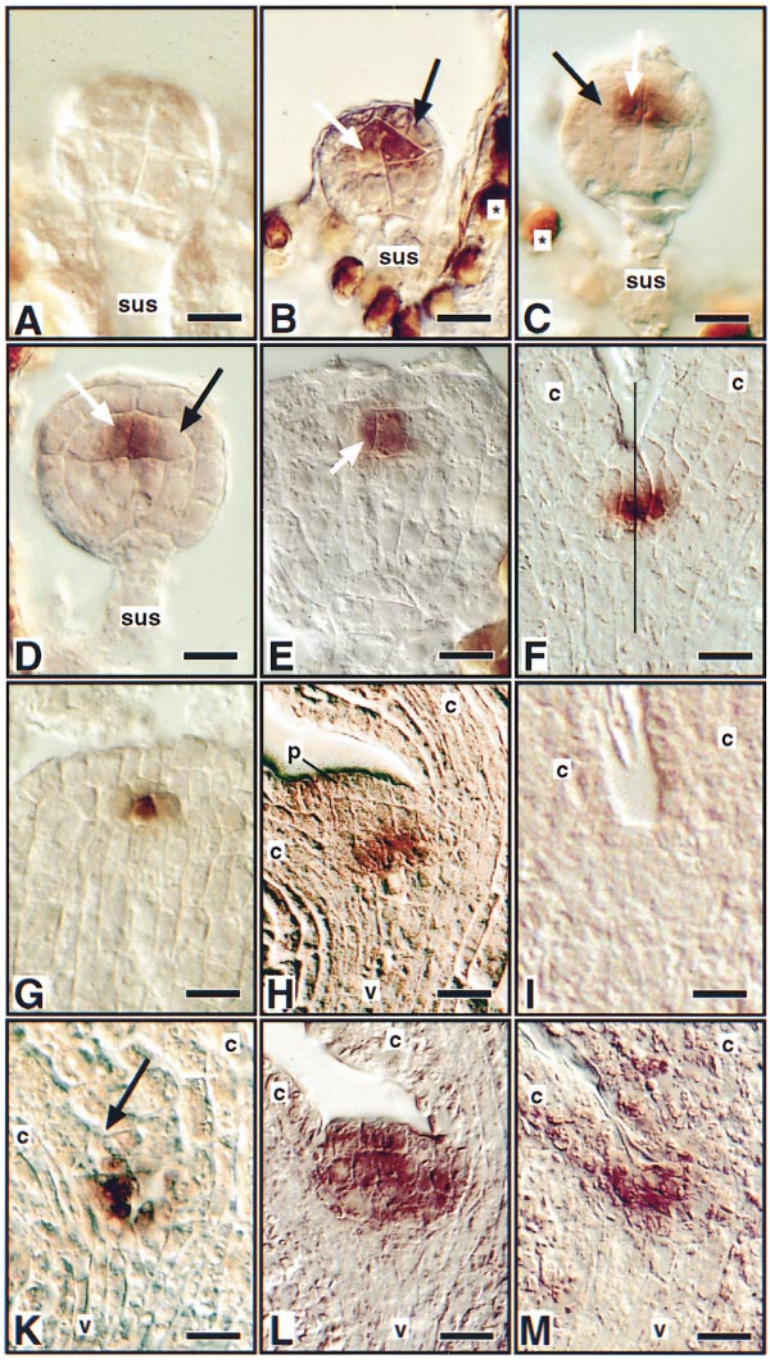

Figure 4. Expression of *WUS* mRNA in *Arabidopsis* Embryo Development

(A) An 8-cell embryo. The four upper cells, two of which are seen in the plane of section, will give rise to cotyledons and shoot meristem, while the four lower cells will form hypocotyl and root. Tissue layers have not formed yet. *WUS* mRNA was not detected at or before this stage.

(B) A 16-cell embryo. All cells of the eight-cell embryo shown in (A) have divided periclinally, separating the outer protoderm and the inner cells. In the inner cells of the apical region (white arrow), *WUS* mRNA is detected, but not in the basal region or in the protoderm (black arrow).

(C) One of the inner apical cells shown in (B) has divided longitudinally. The daughter cell in the center (white arrow) continues *WUS* expression, whereas the peripheral daughter has discontinued *WUS* expression (black arrow).

(D) A 32-cell embryo. All inner apical cells shown in (B) have divided longitudinally. *WUS* mRNA is detected in the central daughter cells (white arrow), but not in the peripheral daughters (black arrow).

(E) Transition-stage embryo. The apical domain contains two cell layers, the protoderm and one subepidermal layer. Two subepidermal cells in the center express *WUS* (white arrow).

(F) Heart-stage embryo. The subepidermal cells shown in (E) have divided transversely, resulting in a three-layered apical domain. *WUS* mRNA was detected in the lower daughters but not in the upper daughters of the *WUS* expressing cells shown in (E). The black line represents the plane of the section shown in (G).

(G) Heart-stage embryo. Only a single cell expresses *WUS* in a longitudinal section as indicated in (F).

(H) Late-stage embryo. A small cell group of the shoot meristem expresses *WUS*. The outer two layers do not show *WUS* expression.

(I) Late heart-stage embryo. Sense control. No staining detected.

(K) Late-stage *stm* embryo. *WUS* expression is found in cells underneath the point where the cotyledons meet (arrow).

(L) Late-stage wild-type embryo. *STM* is expressed throughout the shoot meristem bulge.

(M) Late-stage *wus* embryo. *STM* is expressed in a few apical cells.

WUS mRNA is indicated by dark brown. *, cells of the integument show dark brown color independently of the in situ hybridization. c, cotyledon; v, vasculature; p, leaf primordium; sus, suspensor. Scale bars, 10 μm.

WUS Represents a Novel Type of Homeodomain Proteins

Our data indicate that WUS represents a novel subtype of homeodomain proteins and functions as a transcriptional regulator. The early nonsense mutation in *wus-4* indicates a complete loss of function in this allele. From their indistinguishable phenotypes compared to *wus-4*, we conclude that *wus-1* and *wus-2* also represent null alleles, supporting the previous interpretation of their phenotypic defects (Laux et al., 1996). The less severe *wus-3* phenotype results from an amino acid exchange in the N-terminal part of the homeodomain. The analysis of mutant homeodomains has suggested that this region is essential for DNA binding specificity (Laughon, 1991; Kornberg, 1993). The phenotypic defects in the *wus-3* mutant may thus result from a reduced affinity of WUS for its DNA target(s), consistent with its proposed function as a transcription factor.

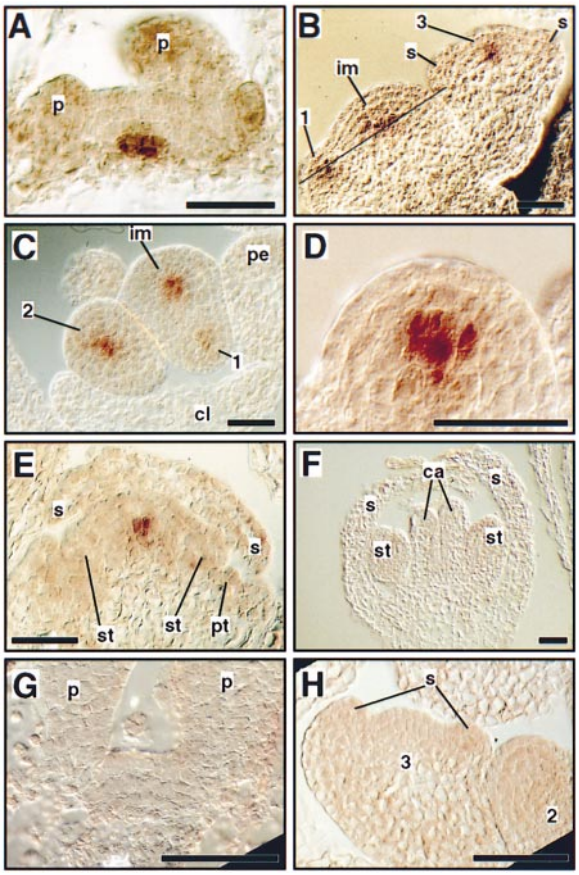

Figure 5. Expression of *WUS* mRNA in Postembryonic Development

(A) Seedling apex. A small group of cells in the center of the shoot meristem, underneath the L3, expresses *WUS*.

(B) Inflorescence. In the inflorescence meristem (im), a small group of central cells underneath the L3 expresses *WUS*. In floral meristems, stages 1 (1) and 3 (3), a small group of cells underneath the L2 expresses *WUS*. Note that the expression domain in floral meristems is shifted one cell layer upward in comparison to vegetative shoot meristems (shown in [A]) and inflorescence meristems. The black line represents the plane of section shown in (C).

(C) Inflorescence. Cross section through an inflorescence (im), a stage 1 (1), and a stage 2 (2) floral meristem all showing *WUS* expression in the center.

(D) Stage 2 flower. Strong *WUS* expression is detected in a cell group below the L2.

(E) Stage 6 flower. Sepals (s), petal (pt), and stamen (st) primordia are visible. *WUS* expression seems to be reduced compared to earlier stages as shown in (D).

(F) Stage 10 flower. Carpel primordia (ca) occupy the center of the flower. No *WUS* expression is detected.

(G) Seedling apex. Sense control. No staining detected.

(H) Stage 2 and 3 flowers. Sense control. No staining detected.

WUS mRNA is indicated by dark brown. p, leaf primordium; pe, pedicel; cl, cauline leaf; s, sepal. Scale bars, 30 μm.

WUS Affects Stem Cell Fate in a Non–Cell-Autonomous Manner

WUS expression defines a novel functional domain of the shoot meristem that has not been previously recognized as a functional or morphological unit. *WUS* is expressed in a group of cells underneath the stem cells, but not in the stem cells themselves, which appear to be misspecified in the *wus* mutant. This finding suggests a model in which *WUS* enables the cells expressing it to function as an organizing center that confers stem cell fate to its overlying neighbors (Figure 6).

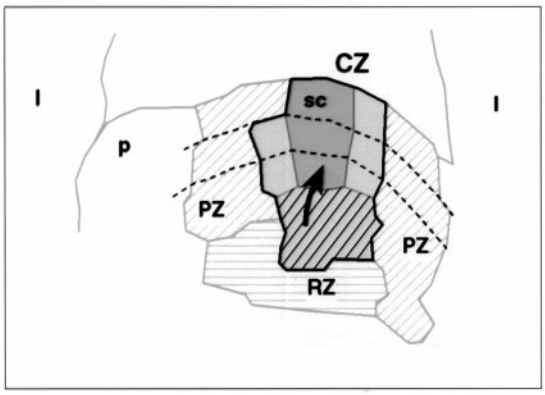

Figure 6. Model for the Role of *WUS* in the Shoot Meristem

WUS expression is confined to a small group of cells (striped) in the lower part of the central zone (CZ, black frame), underneath the L1–L3 stem cells (sc, darkly shaded) and above the rib zone (RZ), a region of flat cells that will form the pith. *WUS*-expressing cells are required to specify the overlying neighbors (arrow) as stem cells. I, leaf; p, leaf primordium; PZ, peripheral zone.

This model suggests common mechanistic principles between shoot and root meristems. The root meristem contains a set of stem cells surrounding a small group of mitotically inactive cells, the quiescent center. The results of ablation studies suggest that the cells of the quiescent center inhibit differentiation of neighboring stem cells (van den Berg et al., 1997). Thus, although there is ample evidence that shoot and root meristems employ different sets of regulatory genes, in both stem cells may be specified by a central organizing center. Both meristems may therefore represent modifications of a basic meristem organization that function in different developmental contexts to give rise to the shoot and the root, respectively. Our findings also suggest similar mechanisms of stem cell specification in plants and animals. For example, in *C. elegans*, the stem cells of the germline are maintained by signaling from a neighboring somatic cell, the distal tip cell (Henderson et al., 1994). Thus, interactions between neighboring cells appear to be a common theme in how different organisms specify the pluripotent state of stem cells. It remains to be determined whether such cellular interactions rely on similar molecular mechanisms.

The phenotypic defects of the respective mutants and the expression patterns of the genes indicate that *WUS* and *STM* act at different regulatory levels in the shoot meristem. *WUS* is expressed in a small subset of meristem cells and specifically affects the fate of cells in the meristem center. By contrast, *STM* is expressed throughout the meristem dome but is downregulated in the incipient organ primordia (Long et al., 1996). Together with its antagonist *CLV1*, it appears to regulate the transition of cells toward differentiation and organ formation, with *STM* preventing premature organ formation (Clark et al., 1996; Endrizzi et al., 1996). Thus, we suggest a model in which *WUS* is required to specify stem cells, while *STM* activity allows their progeny to proliferate before being partitioned into organ primordia.

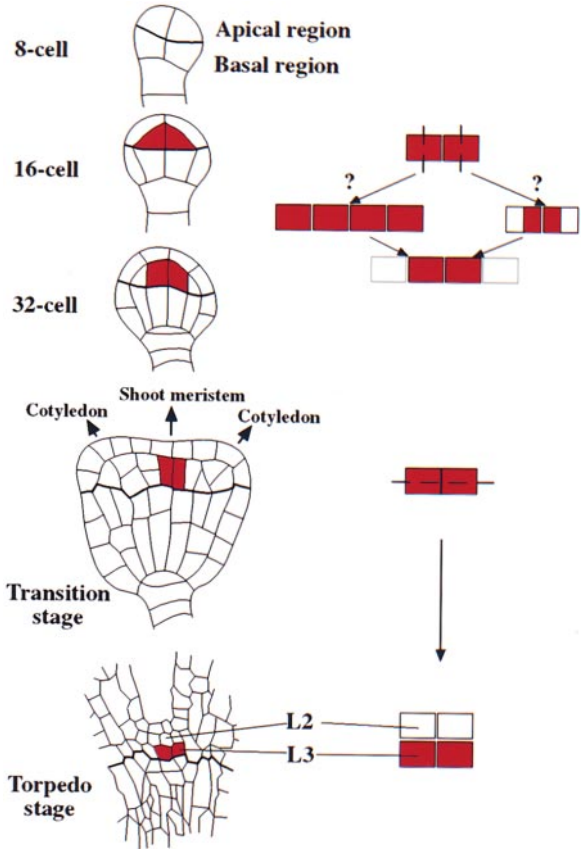

Figure 7. Dynamics of the *WUS* Expression Pattern during Early Embryogenesis

Schematic representation of the *WUS* expression pattern at different embryo stages (left panel) and a diagram of the asymmetric cell divisions that lead to the localization of the *WUS* domain at the base of the shoot meristem primordium. *WUS* expression (red) is initiated in the four subepidermal cells of the apical embryo region at the 16-cell stage. These cells divide longitudinally, but only the central daughter cells continue *WUS* expression. Two mechanisms are depicted: initially all daughter cells inherit *WUS* mRNA, which is subsequently degraded in the peripheral daughter cells, whereas the central daughters sustain *WUS* expression. Alternatively, *WUS* mRNA could already be distributed asymmetrically before cell division and thus would be inherited by only one daughter cell. *WUS*-expressing cells eventually divide horizontally, setting up the L2 and L3 cell layers at the late heart stage. In this case, *WUS* expression is confined to the basal daughter cells. The orientation of the following cell division is indicated by broken lines.

Differences in the Organization of Shoot and Floral Meristems

In contrast to indeterminate shoot meristems, floral meristems terminate due to the recruitment of the central cells for organogenesis. This step is preceded by a downregulation of *WUS* expression, consistent with a role of *WUS*-expressing cells in conferring stem cell fate. It is interesting to note, however, that in floral meristems *WUS* expression is shifted up by one cell layer in comparison to vegetative and inflorescence meristems. Thus, although indeterminate shoot meristems and determinate floral meristems are thought to be homologous structures and share regulatory mechanisms (Endrizzi et al., 1996; Laux et al., 1996; Clark et al., 1997), the *WUS* expression pattern suggests that both meristems are in part organized differently.

Role of *WUS* during Embryonic Shoot Meristem Initiation

The expression of *WUS* in the 16-cell embryo demonstrates that the shoot meristem originates during early embryonic patterning long before it can be recognized histologically (Figure 7). Our data show that at this stage, cell type differences along the apical-basal as well as the radial (inner vs. outer cell layers) axes are already established and that *WUS* expression responds to these differences.

The subsequent changes in the *WUS* expression pattern indicate that embryonic shoot meristem formation is a prolonged dynamic process (Figure 7), the earliest step of which may be the initiation of a group of cells able to specify stem cells. By asymmetric cell divisions, this ability becomes progressively directed to its prospective domain of function in the embryonic shoot meristem primordium. During this process, *WUS* could primarily act in the *WUS*-expressing cells themselves, for example shielding them against differentiation. Alternatively, these cells could already function in a manner similar to the situation in active meristems from the earliest embryo stages on, conferring to overlying neighbors a pluripotent state. However, although in part also derived from the early *WUS*-expressing cells, the cotyledons are not affected in *wus* mutants (Laux et al., 1996). Likewise, the onset of *STM* expression in descendants of the early *WUS*-expressing cells does not require *WUS* activity. These two observations indicate that, although *WUS* is expressed very early in embryogenesis, its requirement is restricted to the development of the proposed organizing center and the specification of stem cells.

Asymmetric cell division is a well-known mechanism to establish different cell fates. For example, in the *C. elegans* embryo, asymmetric localization of the PIE-1 protein marks the germline precursor cells and prevents their differentiation (Mello et al., 1996). In the *Arabidopsis* root meristem, the asymmetric divisions establishing endodermal and cortical cell layers from one set of initial cells require the activity of the *SCARECROW* gene, which is expressed in the initials and their endodermal daughters (Di Laurenzio et al., 1996). The dynamic pattern of *WUS* expression is intriguing in this context, because it may exemplify a more general strategy of how plant embryos direct a specific cell type to its appropriate position, since cell migration, which plays an important role in animal embryogenesis, is precluded in plants.

Conclusions

The analysis of the *WUS* gene leads to novel concepts of how meristems are formed and maintained in plant development. Our data indicate that the formation of a shoot meristem is the outcome of a successive patterning process initiated very early in embryo development. Once the shoot meristem is established, *WUS* expression defines a group of cells that function to specify overlying neighbors as stem cells.

Experimental Procedures

Mutant Lines, Growth Conditions, Histology, and Scanning Electron Microscopy

wus-1, *-2*, and *-3* were induced in the *Landsberg erecta* ecotype. The isolation of *wus-1* and *-2* has been previously described (Laux et al., 1996); *wus-3* was kindly provided by Ulrike Mayer and *wus-4* by Jennifer Fletcher. Plant growth conditions, histology, and scanning electron microscopy were done as previously described (Laux et al., 1996).

Molecular Cloning of *WUS*

For RFLP mapping, 1575 plants from a cross of *wus-1* to *Niederzenz* (*Nd-0*) were examined. DNA was prepared as previously described (Lukowitz et al., 1996). PCR fragments were subcloned using either a T-cloning kit (MBI Fermentas, Vilnius, Lithuania) or the pMOS Blue Vector kit (Amersham). We used the following YAC libraries: yUP (Ecker, 1990), CIC (Creusot et al., 1995), and EW (Ward and Jen, 1990) and a pBIC20 cosmid library (Meyer et al., 1994). YAC end probes were generated by modified vectorette PCR (Matallana et al., 1992) and by TAIL-PCR (Liu and Whittier, 1995). Sequences of CAPS markers, PCR reaction conditions, and primers suitable for yUP, CIC, and EW-YACs are available upon request from the author. The primer pair 5′-TCT CTT GTT CCT CTC TAA GTC TTG-3′ and 5′-CAT AGT TGT GAA CAT ACG AGT ACG-3′ was used to amplify the *WUS* genomic sequence of the mutant alleles. For each allele two products from independent PCR reactions were subcloned and sequenced.

Complementation of *wus-1*

wus-1/WUS plants were crossed twice into the *Ws-2* background. Cosmids containing parts of the *WUS* genomic region were transformed into *Agrobacterium tumefaciens* strain AGL1, and homozygous *wus-1* root explants were transformed as described (Valvekens et al., 1988).

5′-RACE-PCR

5′-RACE-PCR was performed with a 5′-3′-RACE-Kit (Boehringer Mannheim) according to the manufacturer's instructions. Primers used for reverse transcription and PCR reactions were as follows: 5′-GCC TTA TGG TTC TGG AAC CAG-3′, 5′-CTC AAT CTT TCC GAA CTG TCT CAG CC-3′, and 5′-TGA TCG GCT GTT GGT GAC CGG-3′.

Transient Transformation of Onion Epidermis Cells

The construct for transient expression was generated by inserting a PCR-amplified *WUS* cDNA fragment comprising the full-length coding region in-frame with the GUS reporter gene moiety into the vector *pNT160* in place of the Ac moiety (Boehm et al., 1995). For the negative control, the Ac moiety was excised from *pNT160* and the vector religated. Transformation of onion epidermis cells using a PDS1000 helium particle gun (Bio-Rad, Hercules) and histochemical staining were performed as described (Varagona et al., 1992).

In Situ Hybridization

Plant material was fixed with 4% paraformaldehyde (Sigma) in PBS for 8 hr after vacuum infiltration. The tissue was dehydrated and embedded in Paraplast Plus (Oxford Labware). Eight-micrometer sections were placed on SuperFrost/Plus slides (Menzel Gläser). Paraplast was removed by immersion in Histoclear. Sections were rehydrated, incubated 10 min with 0.125 mg/ml Pronase (Sigma) in TE (50 mM Tris-HCl [pH 7.5], 5 mM EDTA), 10 min in 4% paraformaldehyde in PBS, and 10 min in 0.5% acetic anhydride in 0.1 M triethanolamine (pH 8). After dehydration by an ethanol series, slides were air dried before application of the hybridization solution. Per slide, 50–200 ng probe was applied in 80 μl hybridization solution. After incubation in a humid box at 50°C overnight, slides were washed twice in 0.2× SSC for 1 hr at 55°C. After incubation with 20 μg/ml RNAse A for 20 min at 37°C, slides were again washed in 0.2× SSC for 1 hr at 55°C. Slides were incubated in 0.5% blocking reagent (Boehringer) in TBS (100 mM Tris [pH 7.5], 150 mM NaCl) and gently agitated for 45 min. Anti-Digoxigenin-alkaline-phosphatase-coupled antibody (Boehringer) was diluted 1:1250 in BXT (1% BSA, 0.3% Triton X-100 in TBS), 120 μl applied to each slide with a cover slip, and incubated for 2 hr. Slides were then washed 4× 20 min with BXT. One hundered microliters of fresh staining solution (220 μg/ml NBT and 80 μg/ml BCIP in 100 mM Tris [pH 9.5], 50 mM $MgCl_2$, 100 mM NaCl) and cover slips were applied daily for 40–60 hr. For microscopy, 50% glycerol and a cover slip were applied. Photographs were made using a Zeiss Axiophot with Normarski optics and Kodak Ektachrome 64T film. Probes were labeled using Digoxigenin labeling mix (Boehringer) according to the manufacturer's protocol. An antisense probe from a full-length *WUS* cDNA clone was generated using T7 RNA polymerase, and a sense probe was synthesized using T3 RNA polymerase. A 200 bp probe (DraI-XbaI) that contained neither homeobox nor putative transactivation domain led to similar results. In order to exclude cross hybridization with other homeobox genes, we performed low-stringency Southern blot hybridization of *Arabidopsis* genomic DNA with *WUS* full-length cDNA as a probe. In all cases we detected only a single band, indicating that *WUS* cDNA hybridized to a single locus (data not shown). A detailed protocol is available upon request from the author. Protocol modified after Jackson (1991).

Computer Analysis and Image Processing

Predictions of the secondary structure were made using the MacVector software (Kodak Scientific Imaging Systems, New Haven, CT). Database searches were done using the BLAST and BEAUTY algorithms (Altschul et al., 1990; Whorley et al., 1995). A Neighborhood-joining dendrogram was created using the complete-deletion option with the MEGA 1.01 program (Kumar et al., 1994). Image processing was done using the Adobe Photoshop program (Adobe Systems Inc., Mountain View, CA).

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