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Mark P. Running
California Institute of Technology

Elliot M. Meyerowitz
California Institute of Technology

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USING CONFOCAL MICROSCOPY IN THE STUDY OF PLANT STRUCTURE AND DEVELOPMENT

MARK P. RUNNING AND ELLIOT M. MEYEROWITZ

Division of Biology 156-29
California Institute of Technology
Pasadena, CA 91125
Phone: (818) 395-6889, 395-4936
FAX: (818) 449-0756
email: runningm@starbase1.caltech.edu,
meyerowitze@starbase1.caltech.edu

ABSTRACT

The widespread application of confocal laser scanning microscopy has revolutionized biological imaging. We have developed a protocol for using confocal microscopy to examine the development of wild type and mutant *Arabidopsis thaliana,* overcoming the technical difficulties associated with examining whole-mounted plant tissue. This allowed us to rapidly determine the underlying cellular defects that lead to the morphological changes visible in several mutants, and has led to a greater understanding of the mechanisms involved in the control of floral organ number.

Key words: *Arabidopsis,* confocal microscopy, flower development, plant development.

INTRODUCTION

Rapid advances in computer technology have revolutionized biological studies in recent years. In particular, the development of faster, more powerful, and inexpensive computers have enabled the practical application and widespread use of sophisticated imaging techniques. One of these techniques is confocal laser scanning microscopy (CLSM), a specialized type of florescence microscopy. In CLSM, a laser is focused on a single point in a sample, and the resulting fluorescent light is focused on an aperture in such a way that light emanating from above or below the plane of focus falls outside the aperture and is eliminated. Using computer-driven motors, the microscope can scan and obtain image data from a large series of single points in a two dimensional plane, obtaining an optical section through the sample. A series of optical sections can be combined into a three-dimensional image of the sample.

CLSM has many advantages over histological techniques conventionally used in the study of plant anatomy and development, such as manual sectioning and scanning electron microscopy (SEM). CLSM allows image data to be taken from any and all points in a whole mounted sample, and computers can be used to reconstruct the three dimensional image, perform animation and other image processing manipulations to aid in interpretation, and perform virtual sectioning from any angle. Unlike serial sectioning, the integrity of the sample is preserved in CLSM, allowing resampling of data using different parameters. Preparing tissue for CLSM analysis is also much less labor-intensive and much less error-prone than sectioning. SEM can also be used to examine whole-mounted samples, but in SEM only surface cells are visible, whereas in CLSM internal cell patterns can be discerned. CLSM is also very useful for studying tissues that are more inaccessible to dissection, such as the embryo and early floral meristem.

We have used CLSM successfully in our studies of wild type and mutant apical and floral meristem development in a member of the family Brassicaceae, *Arabidopsis thaliana* (Clark et al. 1993, 1995; Running et al. 1995; Running and Meyerowitz 1996). *Arabidopsis* has emerged as a popular model system for genetic and molecular studies of developmental processes in plants (Meyerowitz and Sommerville 1994). The plants are very small, so thousands can be grown at once in a single growth chamber. They are also easy to grow, requiring no special care. In addition, they are self fertile, easy to cross, and have a high fecundity. They also have an exceptionally short generation time (approximately six weeks) and a very small genome size (as small as the nematode *Caenorhabditis elegens* and two-thirds the size of the insect *Drosophila melanogaster*), making genetic characterization and molecular cloning feasible in short time frames.

We are interested in addressing the mechanism of organ initiation in flowers. *Arabidopsis* flowers have a characteristic pattern of four whorls of organs, with four sepals, four petals, six stamens, and two carpels (Smyth et al. 1990). We and others have performed genetic screens to isolate mutants disrupted in this process in order to identify the genes involved. We used confocal microscopy to examine the underlying changes in cell number and pattern in these mutants

in order to identify the primary defect at the early stages of apical and floral meristem development.

MATERIALS AND METHODS

Arabidopsis Mutant Nomenclature

In *Arabidopsis* a gene is given a name and a 2 or 3 letter abbreviation. When referring to the wild type gene, the name and its abbreviation are written in italics and capitals; for example, *PERIANTHIA* (*PAN*). When referring to mutant genes, the name is written in italics and lower case; for example, *perianthia* (*pan*). Sometimes a number of different genes have similar mutant phenotypes; in this case, the genes are numbered sequentially. For example, there are 3 genes that fall into the *CLAVATA* (*CLV*) class, *CLAVATA1* (*CLV1*), *CLAVATA2* (*CLV2*), and *CLAVATA3* (*CLV3*). If there are more than one mutant allele of the gene, each allele is given a number which follows the gene name; examples are *pan-2, clv1-1,* and *clv3-2.*

Plant Material

For all plants, seeds were sown at least 2 cm apart on a 1:1:1 mix of soil:perlite:vermiculite, imbibed at 4 C for 4 days, then placed under 600 foot-candles of constant cool-white fluorescent light at 23 C. Plants were fertilized at regular intervals beginning approximately 7 days after germination. Alleles of *clavata1* (*clv1*) and *clavata3* (*clv3*) were obtained as described (Clark et al. 1993, 1995); alleles of *perianthia* (*pan*) and *hanaba taranu* (*han*) were isolated in a screen of *Agrobacterium tumefaciens*-mediated T-DNA mutagenized *Arabidopsis thaliana* seeds by Kenneth Feldmann at the University of Arizona; *tso1* and *wiggum* emerged from screens of seed lines mutagenized by ethyel methanesulphonate at the California Institute of Technology in the lab of E. M. M.

Confocal Microscopy and Other Imaging

A detailed description of tissue preparation for confocal microscopy is available in Running et al. (1995). Plant tissue is fixed in Formalin:Propionic Acid:Ethanol (10:5:70), placed under vacuum briefly, then left overnight in fixing solution. The samples are then brought through an ethanol series to 100% ethanol to remove chlorophyll and complete the fixing process. After 4 hours in 100% ethanol the sample is brought through a decreasing ethanol series to water, and stained in 5 ug/ml propidium iodide in a solution of 0.1 M L-arginine buffer at pH 12.4 at 4 C for 1 day or more. The samples are then placed in 0.1 M L-arginine buffer at pH 8 without stain for 4 days at 4 C, changing the solution at least once a day. After an increasing ethanol series to 100%, the sample is placed through a series of ethanol/xylene solutions until the sample is in 100% xylene and completely cleared. The sample is dissected (if necessary) in immersion oil, and mounted in a small drop of immersion oil placed on a cover slip. The cover slip is inverted and placed on a depression slide and secured by nail polish. The samples were viewed with a Zeiss LSM10 microscope using an attenuated argon laser emitting at 514 nm as excitation light. We used a Zeiss Neofluar 40× immersion oil lens with a numerical aperture of 1.30. Figures were prepared digitally as described (Sieburth et al. 1995).

RESULTS AND DISCUSSION

Flowers of *Arabidopsis,* like most other members of the Brassicaceae, initiate four sepals, four petals, six stamens, and two carpels in succession (Smyth et al. 1990; Fig. 1A). The organs emerge in a stereotypical pattern: sepals emerge at 90 degree angles around the floral meristem, with two medial and two lateral sepals present. Petals emerge interior to and in between the sepals, with two medial stamens arising interior to each of the medial sepals, and one lateral stamen arising interior to each of the lateral sepals. Carpels emerge in the center of the floral meristem, with the septum forming along the medial axis.

We are interested in the process by which the flower pattern is established: how certain molecular cues tell cells to grow, divide, and differentiate properly according to their position in the flower. As geneticists, our strategy is to identify molecules involved in this process by isolating mutants that are defective in the number or pattern of organs. Study of the mutant phenotype will give us clues into the wild type function of the mutated gene, and in *Arabidopsis* methods to clone genes identified by mutation are available. Our long term goal is to identify mechanisms by which organ number and pattern is determined.

Several mutants affecting organ number have been isolated, all of which affect floral organ number in various ways. The *clavata* (*clv*) class of mutants, comprising three separate loci, show an increase in organ number in all four whorls of the flower, but has the

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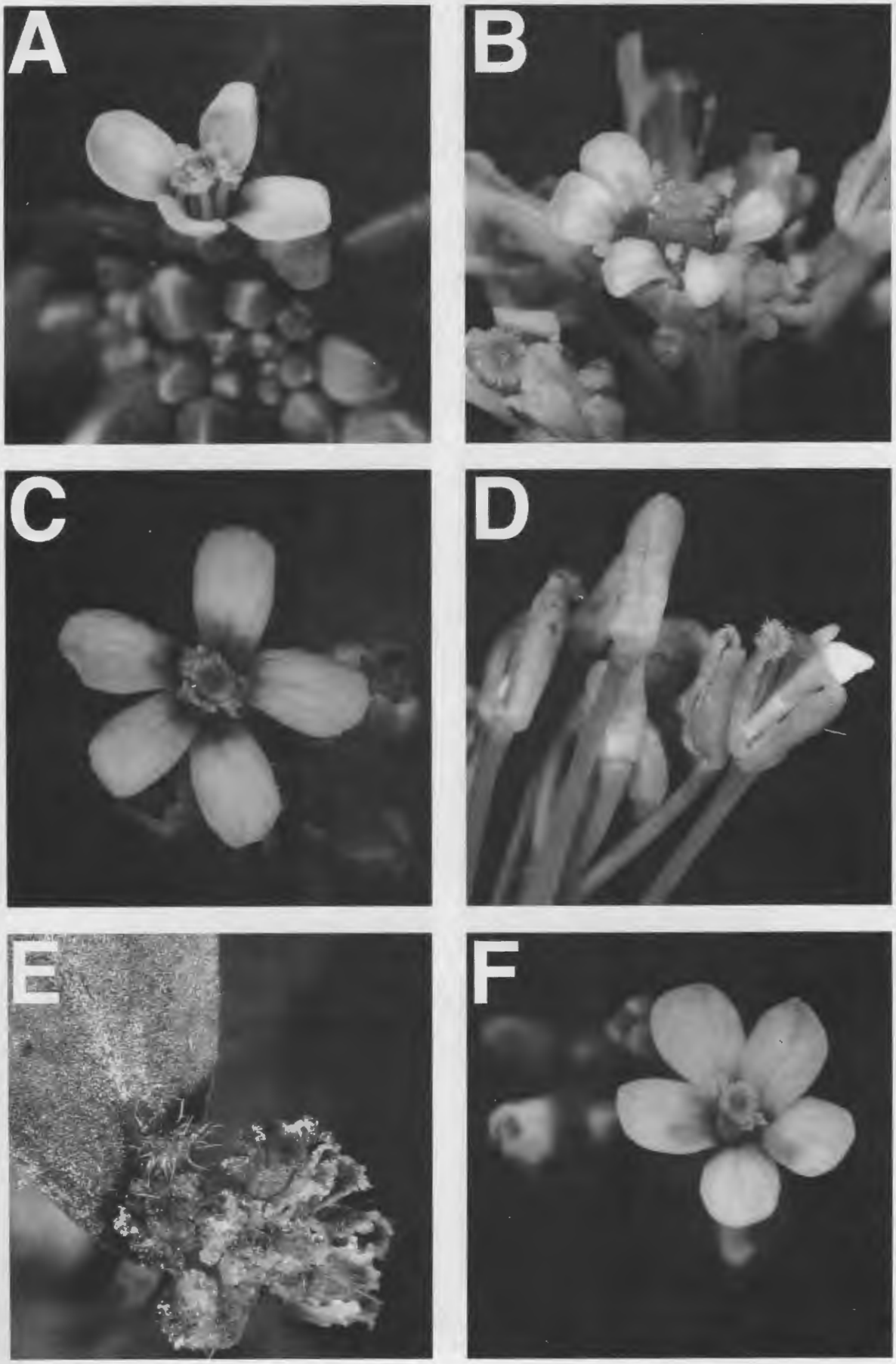
Fig. 1. Wild type and mutant flowers of *Arabidopsis thaliana.*—A. Wild type flower with four sepals, four petals, six stamens, and two carpels.—B. *clavata1-4* mutant flower, with more organs than wild type, especially carpels.—C. *wiggum* mutant flower, with more sepals and petals than wild type.—D. *hanaba taranu* flowers, showing a dramatically decreased number of petals and stamens.—E. *tso1-1* mutant apex, showing flowers that have abnormal sepals and lack interior floral organs.—F. *perianthia* flower with five sepals, five petals, and five stamens.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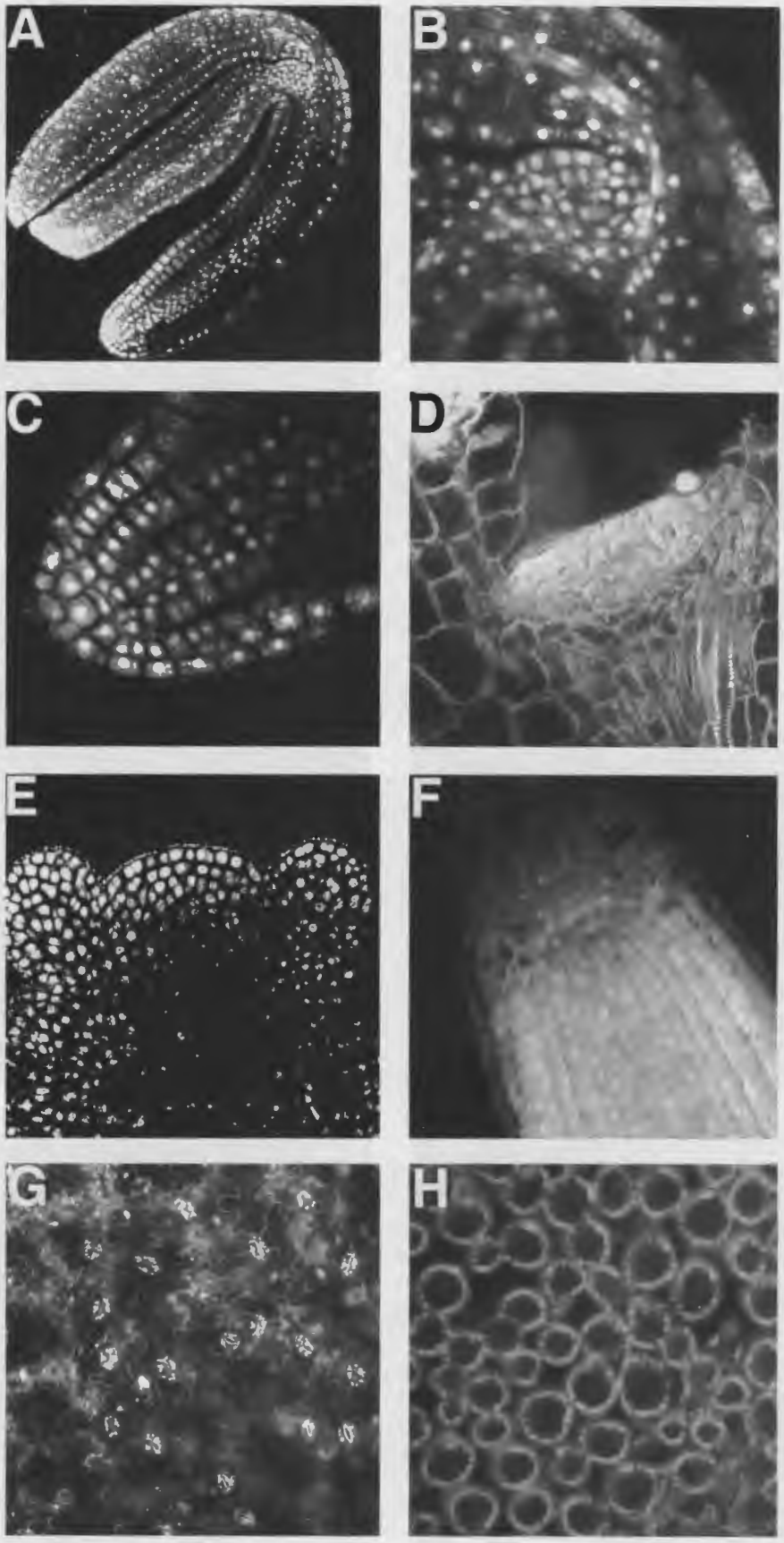

Fig. 2. Examples of wild type *Arabidopsis thaliana* tissues that can be examined using confocal microscopy.—A. Mature embryo, with the seed coat removed.—B. Close-up of the shoot meristem in the mature embryo.—C. Close-up of the root meristem in the mature

most dramatic effect on carpels, and also show additional whorls of carpels growing interior to the fourth whorl of carpels (Fig. 1B; Koornneef et al. 1983; Leyser and Furner 1992; Clark et al. 1993; 1995). The *wiggum* (*wig*) mutant also increases organ number, but its most dramatic effect is on the number of sepals and petals (Fig. 1C; M. P. R. and E. M. M., unpublished). The *hanaba taranu* (*han*) mutant has a different effect: organ number is decreased in all four whorls, but the second and third whorls are most dramatically affected (Fig. 1D; Clark et al. 1994). The *tso1* mutant has a near-normal number of first whorl organs, but second, third, and fourth whorl organs fail to initiate (Fig. 1E; Z. Liu, M. P. R., and E. M. M., unpublished). Finally, *perianthia* (*pan*) mutant flowers tend to have pentamerous flowers (5 sepals, petals, stamens), thus increasing the number of first and second whorl organs but decreasing the number of third whorl organs compared to wild type (Fig. 1F; Running and Meyerowitz 1996).

Once these mutants were isolated, we were interested in examining their development in detail, to see if we could trace back the cause of the defect, or to perhaps correlate it with changes in the earliest stages of the development of the floral apex. In particular, we were interested in seeing how cell number, cell patterns, and meristem size and structure could play a role in determining organ number. We found that examining these mutants with confocal microscopy was the most efficient and effective way of doing this. We developed a protocol that allows nuclear staining of whole mounted plant tissues (Running et al. 1995), and have used it successfully in the examination of many different tissues throughout development (Fig. 2). One stage that is particularly amenable to examination with confocal microscopy is the mature embryo (Fig. 2A), where the shoot apical meristem (Fig. 2B) and root meristem (Fig. 2C) particularly easy to image. The structure of the apical meristem can be followed through development, from the vegetative phase, when it is relatively flat (Fig. 2D), to the adult phase (Fig. 2E), where it becomes more rounded in appearance. The size of the root meristem, and the cell pattern and number in the root, does not change throughout the life of the plant (Fig. 2F). The leaf epidermal (Fig. 2G) and leaf mesophyll cells (Fig. 2H) are also visible under confocal microscopy.

The first locus we examined was *CLAVATA1,* mutations in which lead to plants showing an increase in organ number, particularly in the inner whorls (Fig. 1B). By examining the flowers at the first stage of organ initiation, we could readily detect a size change in the floral meristem (Fig. 3A,B). Specifically, the floral meristem was taller but not wider than wild type at the time the sepals arise. This was due to a greater number of cells, since the size of the cells and the organization of cells into three distinct layers is not affected. The degree of the change in size also correlates with the severity of the organ number defect in different alleles: those alleles with the largest increase in organ and whorl number, such as *clv1-4,* had the greatest increase in early floral meristem size (Clark et al. 1993). *clv3* mutants, which have a phenotype similar to *clv1,* have a similar increase in meristem size (Clark et al. 1995). In examining other tissues in *clv* mutants, we found that the apical meristem also has an increased size, though the size and pattern of cells remains intact (Fig. 3C). Increased meristem size is detectable at least as early as the mature embryo stage (Fig. 3D). Root meristems and other areas of active cell division were not detectably affected.

The *wig* mutant also leads to an increased number of organs in the flower, but its effect is opposite of *clv* mutants in that *wig* primarily affects the outer two whorls, the sepals and petal number showing the greatest deviation from wild type (Fig. 1C). We found that *wig* also has a readily detectable size change in the early floral meristem (Fig. 3E). In this case, though, the meristem is wider but not taller than wild type, which is what presumably leads to extra organs in the first and second whorl.

The mutant *han* shows a decrease rather than an increase in organ number (Fig. 1D). All four whorls are affected, but the second and third whorls show the strongest effect, even lacking organs sometimes. Our CLSM analysis indicates that at the stage where the second and third whorl organs are initiating, the floral meristem size is much smaller in *han* mutants compared to wild type (Fig. 3F,G). In addition, the apical meristem is smaller, visible as early as in the mature embryo (Fig. 3H,I).

Studies of the *clv, wig,* and *han* mutants suggest that one potential mechanism for floral organ number regulation is through regulation of floral meristem size. Another potential mechanism is identified by the *tso1* mutant (Fig. 1E). *tso1* flowers initiate close to the normal number of sepals, but further initiation largely ceases. Instead of petals and reproductive organs, often a mass of undifferentiated tissue appears in the center of the flower. Using CLSM we were able to see that, while the floral meristem size is normal, cell number and pattern are abnormal at the time of sepal initiation (Fig. 3J). Cells are larger and decreased in number,

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embryo.—D. Vegetative shoot apex, which is characteristically flattened.—E. Inflorescence shoot apex, which has a dome shape.—F. Root meristem, which has a stereotypical cell pattern.—G. Leaf epidermis, with some cell wall staining visible; chloroplast DNA in guard cells is brightly stained.—H. Leaf mesophyll; chloroplast DNA staining is especially visible in these cells.

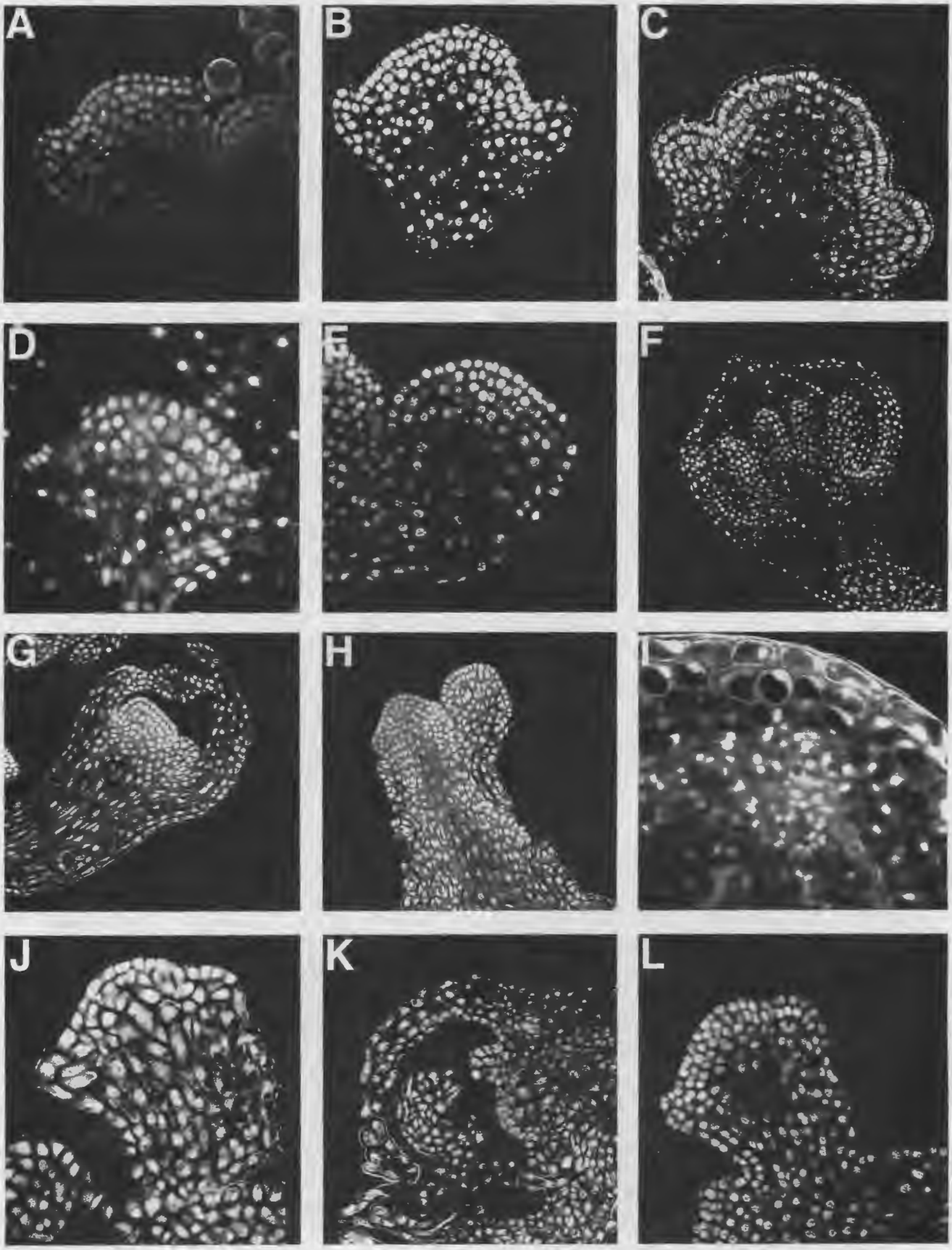

Fig. 3. Confocal microscopy images of wild type and mutant apical and floral meristems.—A. Wild type stage 3 floral meristem. The sepals begin to initiate at this stage.—B. *clv1-1* floral meristem at the same stage as Fig. 3A. The meristem is taller but not wider than

and do not fall into obvious cell layers as they do in wild type. At later stages, the sepals continue to grow, but organ morphogenesis breaks down (Fig. 3K). Many nuclei are large and stain more intensely, indicating that DNA replication continues but nuclear division and cytokinesis are defective. Thus organized cell division patterns are important to some extent in the initiation and development of floral organs.

Another class of genes is represented by *PAN*. The most commonly found *pan* mutant flowers have five sepals, five petals, five stamens, and two carpels, a pattern found in many plant families but not in the Brassicaceae. Unlike the mutants described previously, we were unable to detect a difference in size, cell number, or cell pattern in the floral meristem at the time of sepal initiation (Fig. 3L). Significantly, the organ number increase is more dramatic in *pan* compared to *wig*, in which a size change is readily detectable. Another difference is detectable by SEM: the sepal initiation in *pan* is very regular and predictable, with an adaxial sepal always present, and the rest of the organs equidistant around the ring. In *wig* and the *clv* mutants, sepal initiation is much more irregular.

The histological evidence supports the division of the mutants affecting organ number into three classes: one class that affects meristem size and shape (*clv, han, wig*), one that affects cell division patterns (*tso*), and one that acts specifically at the level of organ initiation (*pan*). Genetic studies also support this classification scheme. Double mutants of genes in different classes have additive phenotypes, and double mutants of genes within the classes have non-additive or synergistic phenotypes. For instance, double mutants between *pan* and *clv* or *pan* and *wig* have about one additional sepal and petal and one fewer stamen compared to *clv* or *wig* alone. The *wig clv* double mutants, though, show dramatically decreased organ number, reduced fertility, and an apex that overproliferates and differentiates into stigmatic tissue.

Our experience has shown that confocal microscopy can be used successfully in the examination of organ number mutants to gain a better understanding of the underlying causes at the cellular level. Conceivably, confocal microscopy could be useful in evolutionary studies in a similar manner. It is possible that changes in floral organ number among closely related species could be due to one or more of the mechanisms suggested by the mutants we have discussed. For instance, a specific size change in the floral meristem will lead to a change in organ number, as in *clv* or *wig* mutants. Similarly, a cessation in cell division in specific regions of the floral meristem may lead to missing organs in particular locations in the flower, as in *tso* mutants. Confocal microscopy is a rapid, convenient method for looking at cell patterns and meristem structure, and could greatly aid such cross-species studies.

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LITERATURE CITED

CLARK, S. E., M. P. RUNNING, AND E. M. MEYEROWITZ. 1993. *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development* **119**: 397–418.

———, ———, AND ———. 1995. *CLAVATA3* is a regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development* **121**: 2057–2067.

———, H. SAKAI, AND E. M. MEYEROWITZ. 1994. Inflorescence development in *clavata* mutants. *In* J. L. Bowman [ed.], Arabidopsis, An Atlas of Morphology and Development. Springer-Verlag, New York, pp. 214–215.

KOORNNEEF, M., J. VAN EDEN, C. J. HANHART, P. STAM, F. J. BRAAKSMA, AND W. J. FEENSTRA. 1983. Linkage map of *Arabidopsis thaliana*. *J. Hered.* **74**: 265–272.

LEYSER, H. M. O., AND I. J. FURNER. 1992. Characterization of three shoot apical meristem mutants of *Arabidopsis thaliana*. *Development* **116**: 397–403.

MEYEROWITZ, E. M., AND C. S. SOMERVILLE. 1994. *Arabidopsis*. Cold Spring Harbor Laboratory Press.

RUNNING, M. P., S. E. CLARK, AND E. M. MEYEROWITZ. 1995. Confocal Microscopy of the Shoot Apex. In D. W. Galbraith, D. P. Bourque, and H. J. Bohnert [eds.], Methods in Cell Biology, Vol. 49. San Diego: Academic Press, pp. 217–229.

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wild type, and produces more inner whorl organs.—C. *clv1-1* inflorescence apical meristem. Compared to wild type (Fig. 2E), *clv* apical meristems have more cells and are taller.—D. *clv1-4* apical meristem in the mature embryo. At this stage *clv* mutants already have more apical meristem cells than in wild type (Fig. 2B).—E. *wig* floral meristem at stage 2, before sepals are clearly visible; the meristem is wider but not taller than wild type.—F. Wild type floral meristem at stage 6. In this section the sepals that enclose the bud are visible, as well as two stamen and two carpel primordia.—G. *han* floral meristem at stage 6. The flower is much narrower than wild type at this stage, leaving little room for interior organ initiation.—H. *han* inflorescence apical meristem. The apical meristem is also much narrower than wild type (Fig. 2E).—I. *han* apical meristem at the mature embryo stage. In many mature embryos the apical meristem is not readily visible, and is invariably smaller than wild type—J. *tso1-1* stage 3 floral meristem. The sepals have initiated, but the cells are much bigger than wild type—K. *tso1-1* stage 6 floral meristem. Cells continue to proliferate, but organ development is disrupted.—L. *pan-2* floral meristem at stage 3. *pan* floral meristems are indistinguishable from wild type in size, cell pattern, and cell number.

———, AND E. M. MEYEROWITZ. 1996. Mutations in the *PERIANTHIA* gene of *Arabidopsis* specifically alter floral organ number and initiation pattern. *Development* 122: 1261–1269.

SIEBURTH, L. E., M. P. RUNNING, AND E. M. MEYEROWITZ. 1995. Genetic separation of third and fourth whorl functions of *Agamous*. *Plant Cell* 7: 1249–1258.

SMYTH, D. R., J. L. BOWMAN, J. L. AND E. M. MEYEROWITZ. 1990. Early flower development in *Arabidopsis*. *Plant Cell* 2: 755–767.