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Research

The genetic control of plant development is mediated by cellular interactions, and an exchange of positional information contributes to the self-organisation and coordination of cells during development. The *Arabidopsis* root meristem provides an ideal test-bed for probing these interactions. The root meristem grows indeterminately, is genetically amenable, has a simple and transparent architecture, and can be induced to form *de novo* in adult tissues. We have developed new genetic and optical techniques for following organisation of cells within living meristems, using a modified green fluorescent protein. We have generated a large library of *Arabidopsis* lines that can be used for targeted gene expression in the root meristem. We can precisely visualise and genetically manipulate particular cells within a living meristem, and we have initiated cell ablation and misexpression experiments in order to gain a better outline of the cellular interactions that underlie organisation of the root meristem.

In order to better interpret these experiments, it is crucial to gain an improved understanding of the precise timing and arrangement of cell divisions within normal and perturbed meristems. We have aimed to develop better techniques for the three dimensional visualisation of cell proliferation during initiation and growth of root meristems. We already have a unique set of fluorescent markers for particular cell types, and have established simple techniques for high resolution 3D imaging of *Arabidopsis* root tissues using confocal fluorescence microscopy. It is now feasible to use computer visualisation methods to reduce large data sets to a simple description of the 3D shapes and arrangement of cells in a meristem. We have adapted 3D segmentation methods for marking the arrangements of particular cells within optically sectioned meristems. We have produced software for improved 3D segmentation. The first method involves the inflation of deformable meshes within the dataset at chosen seed points. This proves to be a robust form of segmentation that is much less sensitive to noise in the experimental data and produces a compact description of cell shapes directly. The second segmentation module is a watershed based algorithm that allows quantitation of the number and area of shared walls between an individual cell and all of its neighbours. These values are highly relevant biologically as they describe the cell connectivity, and correspond to possible avenues for the exchange of informational molecules that regulate cell behaviour – and are thus an important parameter for dynamic modelling approaches.

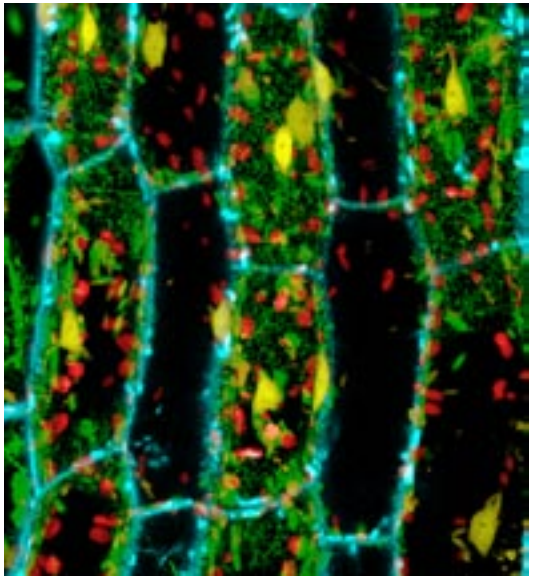
In the first steps towards developing a system for dynamic modelling of plant cell interactions, we have generated a 2D description of the physical properties of cells, using a novel double spring model to describe cell wall properties. This physical model provides an engine for the production of cells through enlargement and division. Fields of proliferating cells can then be programmed *via* a genetic script to produce and respond to different morphogens. We are using this 2D system as a pilot study for modelling cell dynamics and interactions in 3D. It is already proving very useful as a tool for modelling interactions within single layers of cells, such as the epidermis, and for modelling zonation within a meristem.

Contents

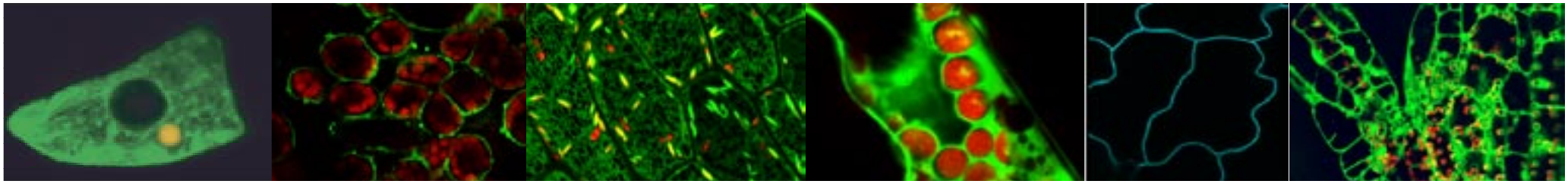
Fluorescent protein markers in Arabidopsis plants.

Over past years, we have developed a set of new genetic and optical techniques which allow us to visualise and manipulate cells within living plants. Soon after the cloning of the *Aequoria victoria* jellyfish green fluorescent protein (GFP) by Douglas Prasher, we obtained the cDNA sequence and proceeded to adapt the gene for use as a bright marker in transgenic plants. Unfortunately, the wild-type GFP cDNA is not expressed in *Arabidopsis*. We identified and removed a cryptic intron from within the *gfp* gene, and introduced modifications that confer improved folding and spectral properties and to alter the subcellular localisation of the protein. All of these alterations were incorporated into a single modified form of the gene (*mgfp5-ER*) which we now routinely use for monitoring gene expression and marking cells in live transgenic plants (Siemering *et al.*, Current Biology 6:1653-1663, 1996; Haseloff *et al.*, PNAS 94:2122-2127,1997).

We have developed fluorescence microscopy techniques for high resolution observation of living cells. The expression of GFP within an organism produces an intrinsic fluorescence that colours normal cellular processes, and high resolution optical techniques can be used non-invasively to monitor the dynamic activities of these living cells. Using coverslip-based culture vessels, specialised microscope objectives and the optical sectioning properties of the confocal microscope, it is possible to monitor simply and precisely both the arrangement of living cells within a meristem, and their behaviour through long time-lapse observations. In order to produce different coloured fluorescent proteins that are expressed well in plants, we have produced the variants mYFP (S65G, S72A, V163A, I167T, S175G, T203Y) and mCFP, (Y66W, V163A, S175G). The mCFP and mYFP variants have properties comparable to mGFP5, but possess distinct spectral properties. Although the emission spectra of the fluorescent proteins overlap to a large degree, laser scanning confocal microscopy allows the use of monochromatic light for selective excitation of the GFP variants. In addition, we are using derivatives of fluorescent proteins from coral to provide longer wavelength signals.



We have generated a range of GFP protein fusions that have provided useful subcellular markers in *Arabidopsis*. For example, we have produced histone2B and extensin fusions that are useful for following nuclear dynamics and visualising cell wall substructure, respectively.

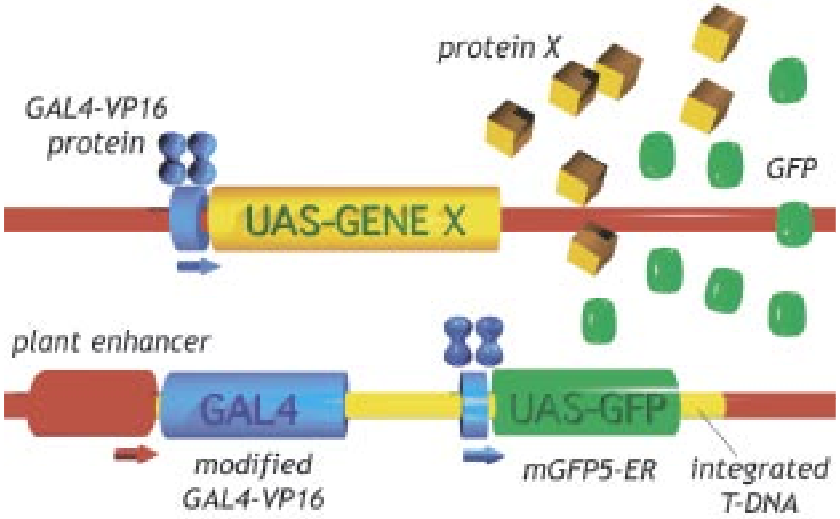


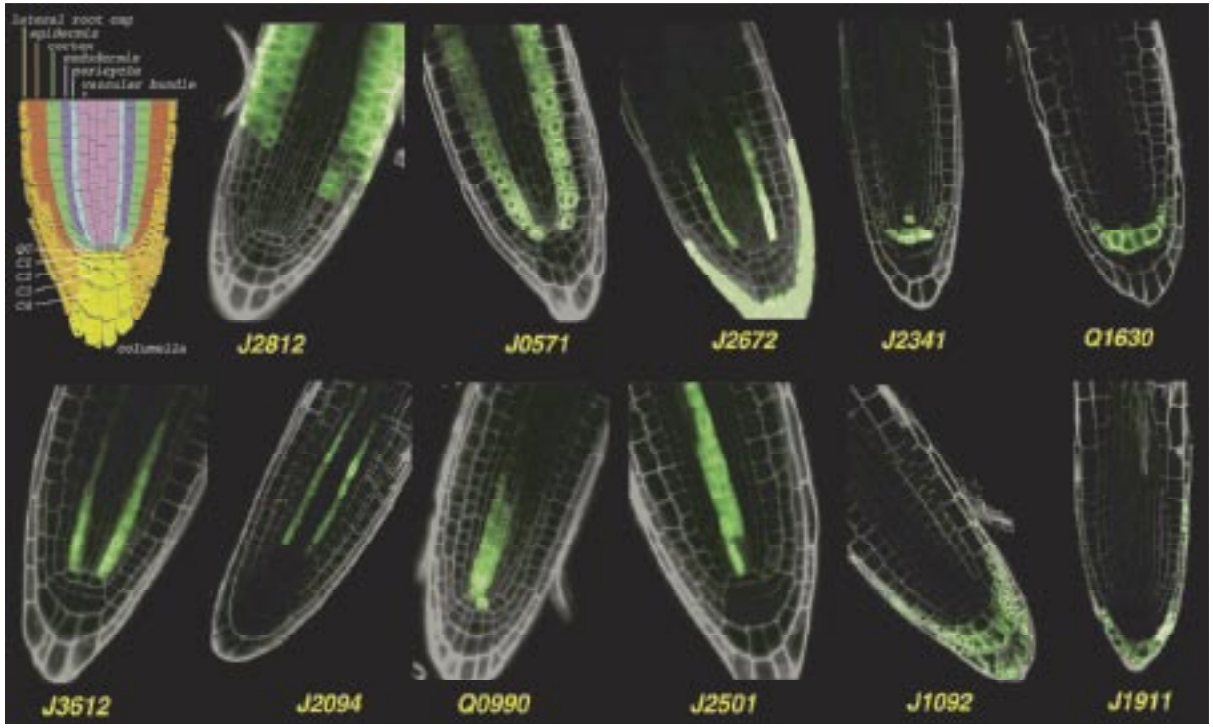
GAL4 targeted expression.

In order to genetically manipulate cells during meristem development, we have devised a scheme for targeted gene expression, which is based on a method widely used in *Drosophila* (Brand and Perrimon, Development 118:401-415, 1993). We have used an "enhancer-trap" strategy to generate many transgenic plants which express different patterns of a yeast transcription activator, GAL4. A chosen target gene can then be placed under the control of GAL4 upstream activation sequences (UAS), transformed into plants, and maintained silently in the absence of GAL4. Genetic crosses between this single line and any of the library of GAL4-containing lines specifically activates the target gene in a particular tissue or cell type. The phenotypic consequences of mis-expression, including those deleterious to the organism, can be conveniently studied.

We found that GAL4 is not expressed in *Arabidopsis* due to a high A/T content, which can interfere with mRNA processing in plants. We have produced a modified form, mGAL4-VP16, that it is expressed efficiently in plants, and randomly inserted the modified gene into the *Arabidopsis* genome, using *Agrobacterium tumefaciens*-mediated transformation. The transformation vector was designed so that expression of the mGAL4-VP16 gene would be dependent upon the fortuitous proximity of an *Arabidopsis* enhancer element. The inserted DNA also contained a GAL4-responsive *mGFP5-ER* gene. Thus, interesting "enhancer-trap" patterns of GAL4 gene expression were immediately and directly visible, with each GAL4-expressing cell marked by bright green fluorescence. We have used in vivo detection of GFP to directly screen for GAL4-directed GFP expression in 8000 regenerated plantlets. We have documented a collection of over 250 *Arabidopsis* lines with distinct and stable patterns of mGAL4-VP16 and GFP expression in the root. This collection has been added to by a screen of an extra 5000 lines generated as part of a collaboration with Scott Poethig's laboratory. In addition, we have recently completed the generation of another 13,000 lines containing a HAP1-CFP enhancer trap vector. These libraries of transgenic lines provide several major benefits:

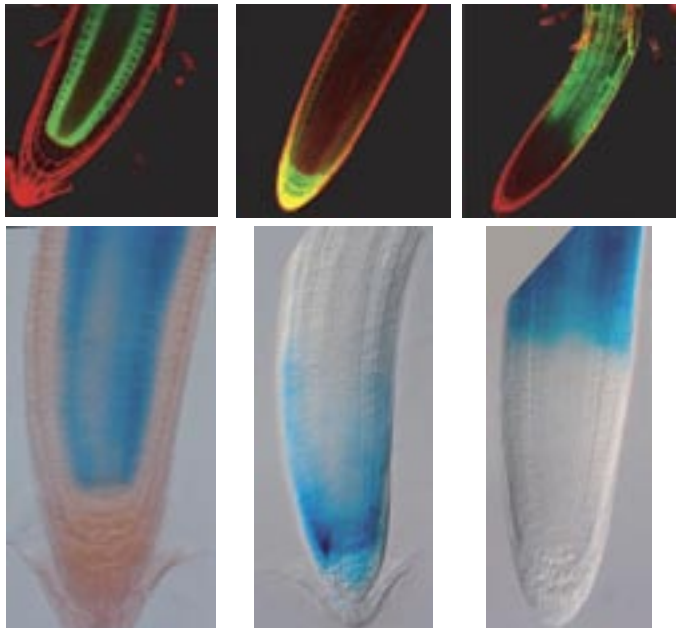
(i) a valuable set of markers, where particular cell types are tagged and can be visualised with unprecedented ease and clarity in living plants. These are





becoming widely used in developmental studies, e.g. Berger *et al*., Current Biology, 1998; Sabatini *et al*., 1999; Wysocka-Diller *et al*., 2000.

(ii) a means of triggering ectopic expression of a chosen gene at a particular time and place during *Arabidopsis* development. Regulatory proteins or toxins can be transformed and maintained in *Arabidopsis*, silent behind a GAL4-responsive promoter. We can now activate these genes in specific cells by crossing to a chosen mGAL4-VP16 expressing line. This is a powerful system for genetically perturbing and monitoring the behaviour of particular cells within a living plant.



(iii) a source of mutants. The collections contain transformed lines with mutant phenotypes, some likely T-DNA tagged. We have also used the GAL4 system as the basis for a gain-of-function screen in the root epidermis, and have islolated a number of mutants including a tagged allele of *tornado1*. (L. Laplaze, E. Truernit & J.H. unpublished).

(iv) a means of identifying important regulatory genes. Several GAL4 lines with bright and distinct GFP expression patterns have been mapped and found to identify important regulatory genes. For example, we have found that the GAL4 gene in line J0481 is inserted next to a WRKY transcription factor expressed in the root epidermis (L. Laplaze & J.H. unpublished), and similarly M0223 contains an insertion adjacent to the *Cup-Shaped Cotyledon 1* gene (Cary et al., 2002).

Visualisation of cell interactions in plant meristems.

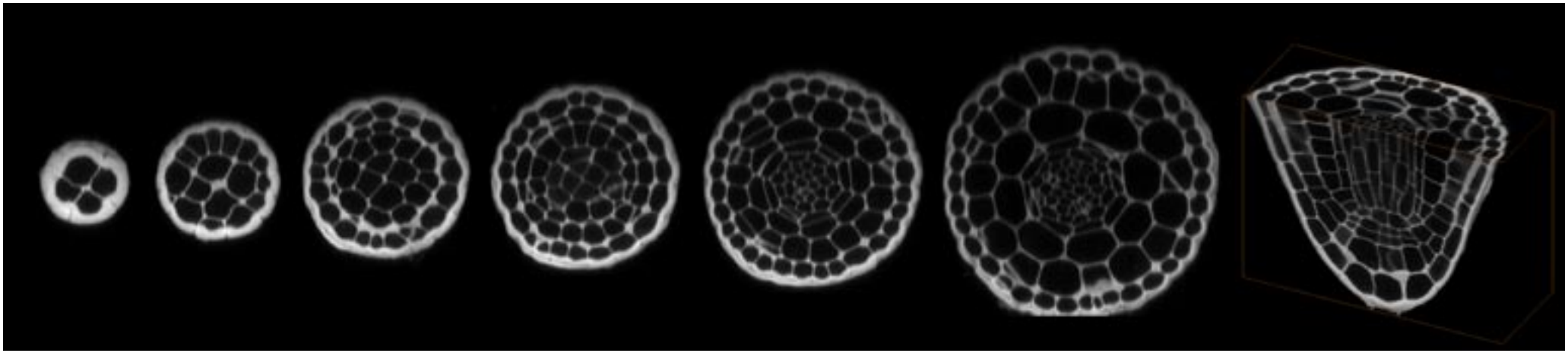
3D microscopy.

The 3D cellular architecture of meristems is difficult to observe in intact tissue as details are obscured by overlying layers of cytoplasm and cell wall. The anatomical arrangements of plant cells have conventionally been observed using microtomy techniques. However the laborious nature of thin sectioning, the problem of obtaining the desired plane of section, and difficulty of obtaining a complete series of sections has limited its use somewhat to the skilled and patient. Optical sectioning has many advantages from the point of view of speed and simplicity, and it can allow the direct viewing of living wholemounts. Here, transverse sections need to be reconstructed from a series of Z-axis images. Nomarski optics have proved useful for examining details within living tissues, but do not provide sufficient contrast and resolution to allow precise 3D reconstruction of cell arrangements. Confocal laser scanning microscopy provides a substantial improvement, but it has still proved difficult to optically section deep into tissues due to light scattering and spherical aberration caused by particulate subcellular matter and layers of refractile cell walls.

We have developed new techniques for intense, specific staining and clearing of plant architecture for 3D microscopy (Bougourd *et al*., 2000; Haseloff, unpublished). For example, periodic acid treatment of carbohydrates produces aldehyde groups which can be reacted with various fluorescent pseudoSchiff reagents. If fixed plant tissue is treated in this way, cell walls (and starch-containing plastids, if present) become intensely and covalently labeled with the fluor. The tissue can then be directly cleared in a high refractive index agent containing chloral hydrate, and mounted for microscopy. The combination of high levels of fluorescence and high refractive index mountant allows the collection of extended Z-series images at very fine resolution (0.2 - 0.5 µM steps), using minimum confocal aperture, and without fear of photobleaching or signal and resolution loss due to spherical aberration. The depth of image collection is limited mainly by the working distance of the objective (~200µM), and this allows simple optical sectioning throughout an entire *Arabidiopsis* root at high resolution. In fact every cell within a mature Arabidopsis embryo can be clearly visualised.

3D segmentation.

We have adapted 3D segmentation techniques (similar to those used in medical imaging) to allow rapid visualisation of cell arrangements within intact embryos and meristems. We use AMIRA, a general purpose physical modelling and data visualisation software package, for visualising large multidimensional confocal microscopy datasets. It provides a very useful set of input/output, data handling and viewer modules. AMIRA allows software routines to be combined in a modular fashion to allow specialised 3D reconstruction and visuali-

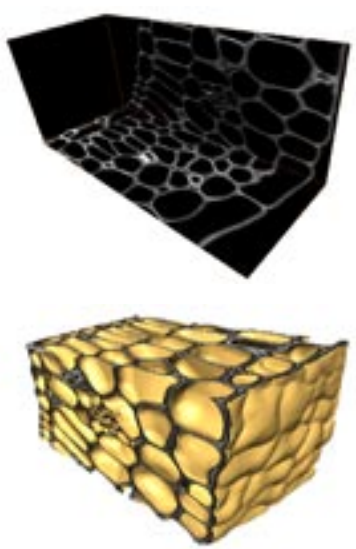


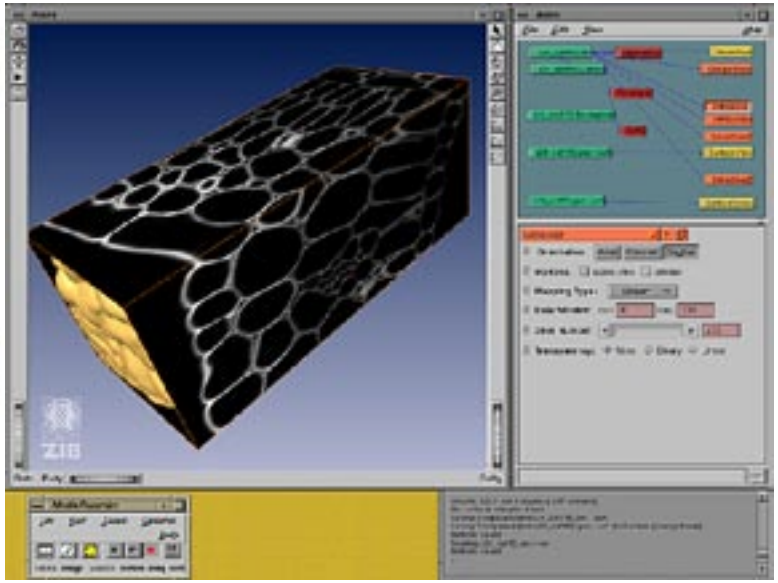
sation techniques to be applied to confocal data. It provides a simple interface, sophisticated, fast visualisation routines, is affordable and robust, and is suitable for both high-end PC and UNIX hardware.

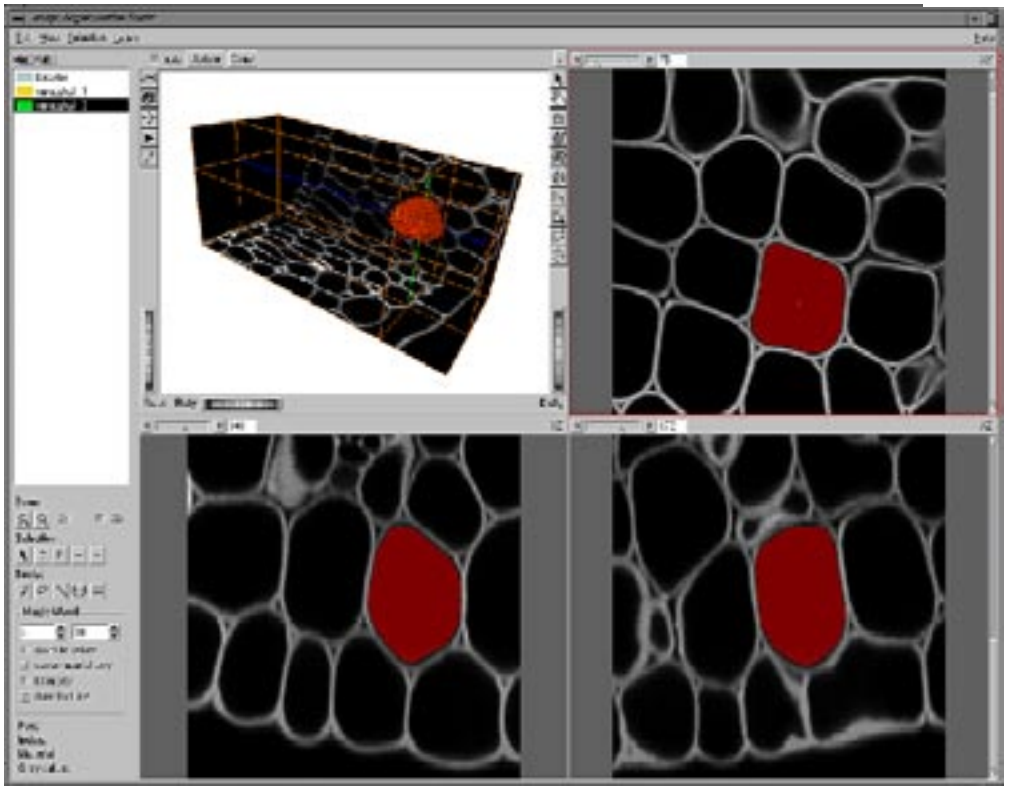
In the first step, *Arabidopsis* tissues are stained using a pseudo-Schiff reaction to specifically label carbohydrate groups in cell walls, and are mounted in a chloral hydrate based clearing agent for microscopy. The combination of clearing and intense staining allows deep optical sectioning using a laser scanning confocal microscope.

A series of confocal optical sections (100-200 MB) is loaded into the AMIRA visualisation software. The package provides software modules for I/O, data slicing, mapping, segmentation and the rendering of surface and solid geometries.

The AMIRA 3D segmentation editor can be used to seed and label particular voxels that correspond to a chosen plant cell within the confocal dataset. The use of a specific cell wall stain allows easy selection of the internal volumes of individual cells. A closed triangulated surface can be formed over the selected group of voxels, using a marching cubes algorithm.







The surface rendered cell is displayed at the correct position and scale within the dataset. It has been transformed from an unconnected group of voxels to a single object which can be manipulated at will.

Repeating the process builds an accurate representation of the shapes, arrangement and connectivity of cells within the tissue.

We can now routinely reconstruct the cellular structure of entire meristems for various experiments. The large data files, up to 1 GB in size, can be directly rendered to allow excavation of the data, production of sections in arbitrary planes, and rendering of surface features. We use computer visualisation methods, adapted from the medical imaging field, to reduce large data sets to a simple description of the 3D shapes and arrangement of cells in a meristem. AMIRA provides advanced software methods for 3D segmentation that in turn allow a geometric description of the dimensions, shapes and relative arrangements of cells within optically sectioned meristems. These same methods can

be used to analyse meristems that have been genetically perturbed by GAL4 targeted cell ablation or misexpression.

New segmentation methods.

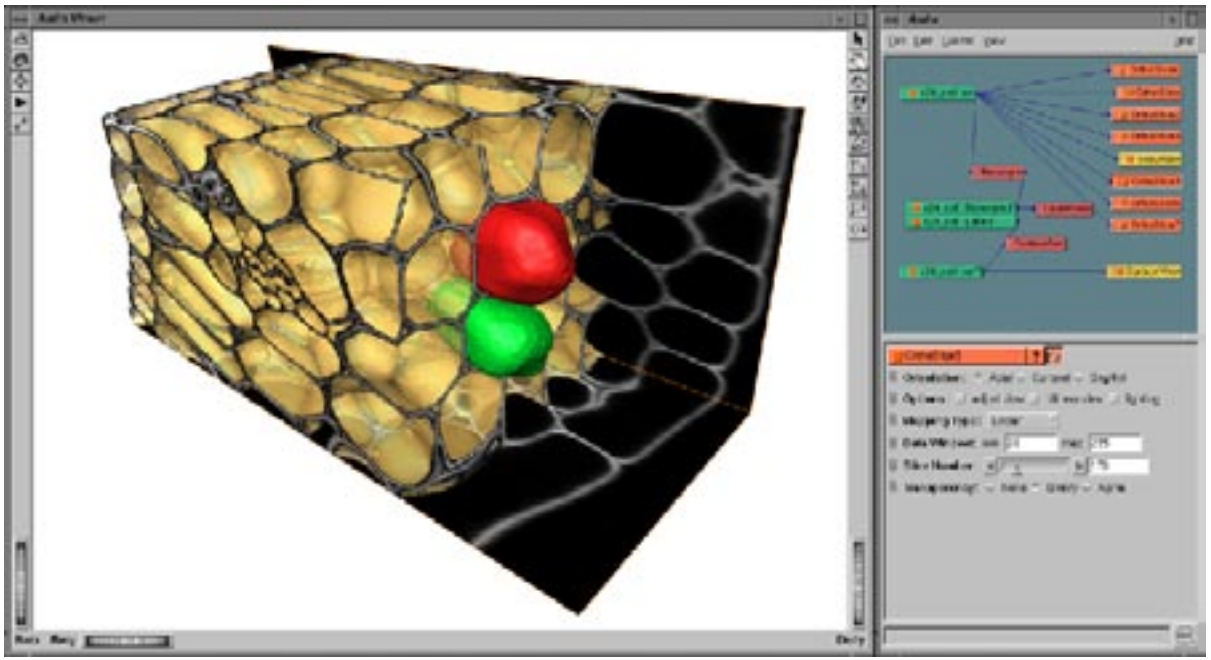
We are developing improved software approaches for the 3D visualisation of cells in plant meristems. These techniques will provide a basis for the dynamic modelling of meristem development.

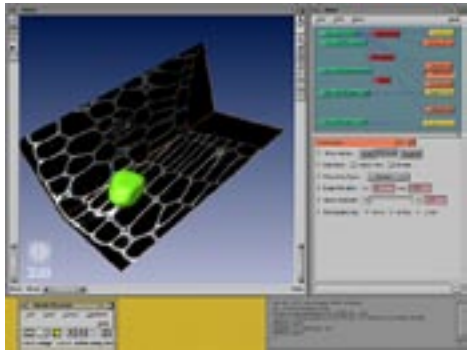
Deformable mesh 3D segmentation.

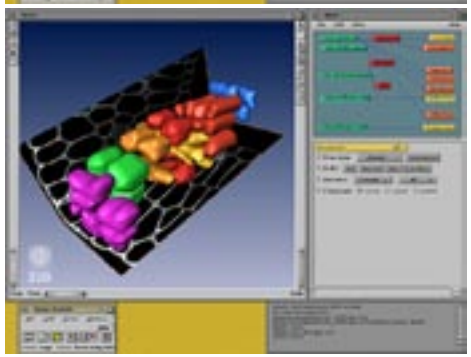
We have produced two modules for improved 3D segmentation in AMIRA. The first method involves the inflation of deformable meshes within the dataset at chosen seed points. This proves to be a robust form of segmentation that is much less sensitive to noise in the experimental data and produces a compact description of cell shapes directly (other techniques produce an intermediate segmented volume, from which a surface must be generated and smoothed). This module will be extended to allow automatic segmentation of an entire collection of cells from a single seed point, and we wish to apply it to live, GFP labelled tissues.

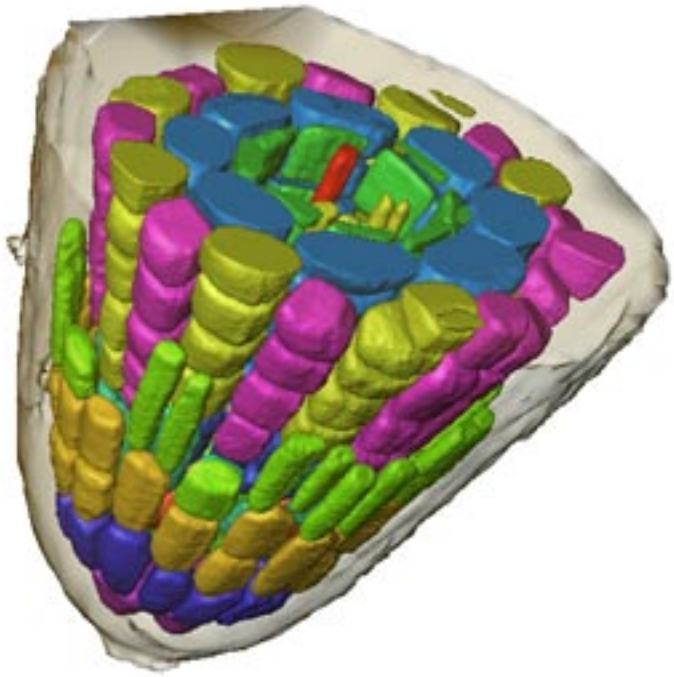
Watershed 3D segmentation.

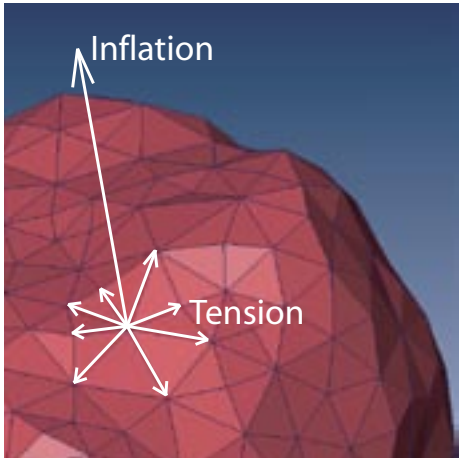
The second segmentation module includes a watershed based algorithm that allows the determination of connectivity between adjacent cells within an intact tissue. For the first time it is possible to automatically obtain a measurement of the number and area of shared walls between an individual cell and all of its neighbours. These values are highly relevant biologically as they correspond to shared walls that contain plasmodesmata, which provide conduits for informational molecules that regulate cell behaviour – and are thus an important parameter for modelling approaches.

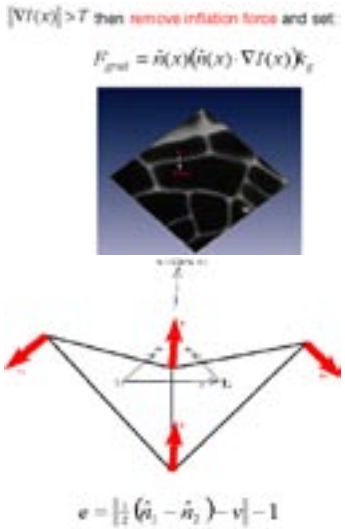












Deformable mesh algorithm.

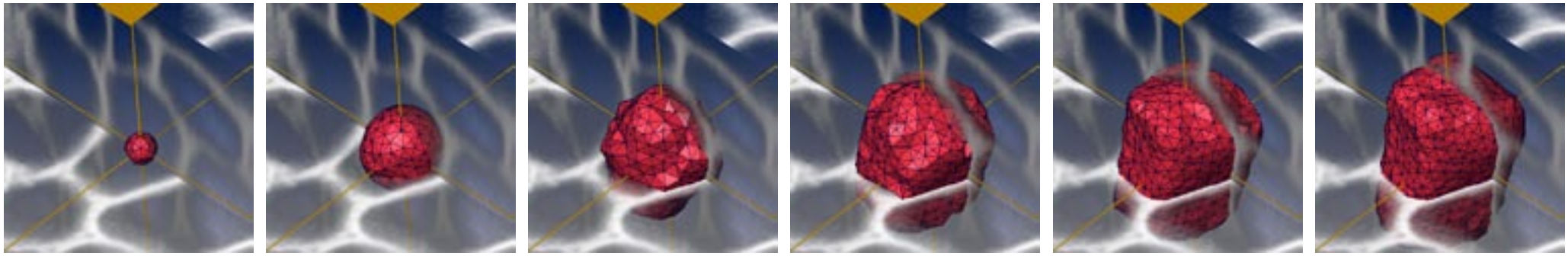
We can obtain 3D confocal images of plant specimens with fluorescently stained cell walls. These give a single channel intensity image with high intensity at the boundary of the cell volumes and relatively flat intensity in the cell interiors. The cells we are particularly interested in (those of the root meristem) are generally convex and have simple shapes. We have developed an algorithm which, given initial points in the interior of a set of cells, produces triangle mesh representations of the specified cells. The algorithm is based on similar work by Boden et al. (1997) and McInerney & Terzopoulos (1996). These methods simulate an elastic surface expanding from the interior of the object to be segmented and stopping at the boundaries. The surface evolves through a discrete time physical model and adaptively subdivides to fit the object boundary.

Physical Model.

Expansion: The elastic surface is simulated by considering each edge as a spring and damper. The springs have some stiffness and natural length zero, meaning they are always in tension. A constant inflating force is applied to each vertex in the direction of the outward surface normal. In addition, we restrict surface curvature. The forces resulting from inflation, tension, bending and damping are accumulated at vertices and the system is iterated until the root mean square velocity of the vertices is below a threshold or until a specified maximum number of iterations have been completed. **Stopping*:*** We use a force based on the image gradient vector to bring the surface to rest at the cell boundaries. When the gradient force is significant the inflation force is removed. At this point the mesh is assumed to have found the boundary and is allowed to follow it. This means that vertices are attracted to intensity maxima, which correspond to the middle of the wall dividing a cell from its neighbour.

Mesh Generation.

An initial mesh consisting of a closed triangulated surface centred on each seed point is created near the centre of a cell. Adaptive subdivision is performed after each time-step of the physical model. This ensures that the mesh remains evenly triangulated as the surfaces expand to fill the cell shapes.



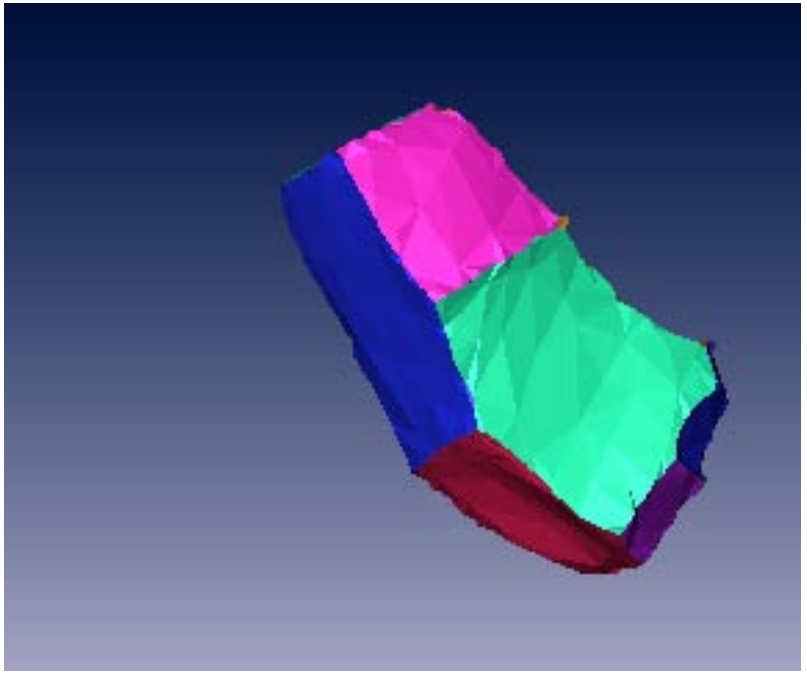
Watershed algorithm for segmentation.

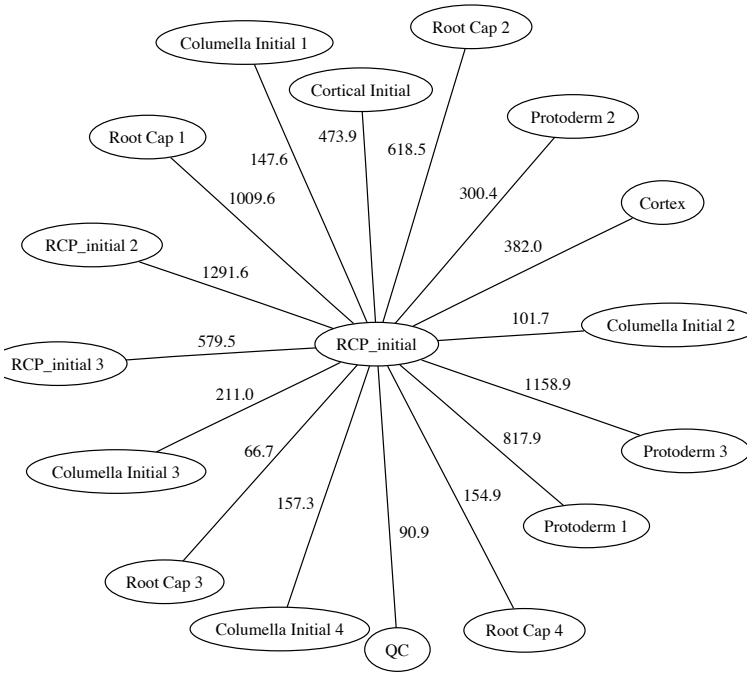
The watershed algorithm is based on the metaphor of water catchment basins in a landscape. The algorithm proceeds in several steps. First, an initial classification of all points into catchment basin regions is done by tracing each point down its path of steepest descent to a local minima. Next, neighbouring regions and the boundaries between them are analyzed according to minimum boundary height to produce a tree of merges among adjacent regions. Metaphorically, the flood level is a value that reflects the amount of precipitation that is rained into the catchment basins. As the flood level rises, boundaries between adjacent segments erode and those segments merge.

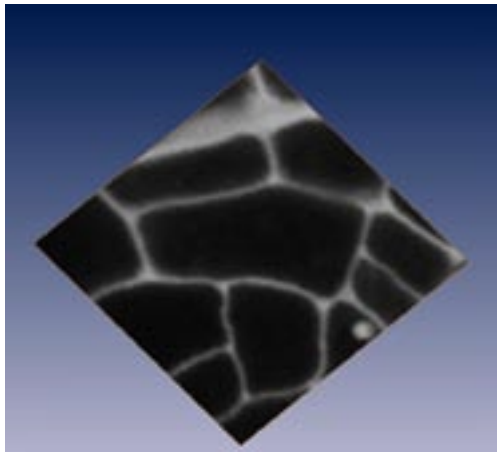
We have adopted code from the National Library of Medicine Insight Toolkit (ITK), to provide a watershed algorithm for the TGS Amira visualisation platform.

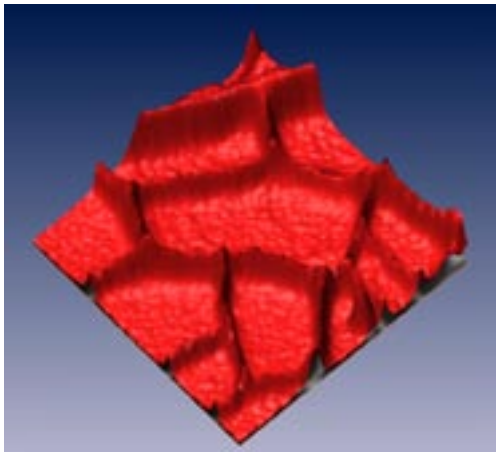
We use the confocal image intensity as the landscape height. This gives ridges between cells and slightly uneven terrain in the cell interiors. By changing the flood level we can interactively traverse the merge tree and thus fine tune the segmentation to accurately include as many cells as possible.

A major advantage of this method is that it produces adjacent segmentations of cells. That is, there is a single boundary between cells with no empty unclassified space between them. We can use this property to compute an adjacency network for the cells and measure cell-cell contact areas. This can then be represented diagrammatically using graph generation software (AT&T Graphvis). It also gives us an excellent starting point from which to extract a simple mathematical representation of cell matrices from real plant specimens.



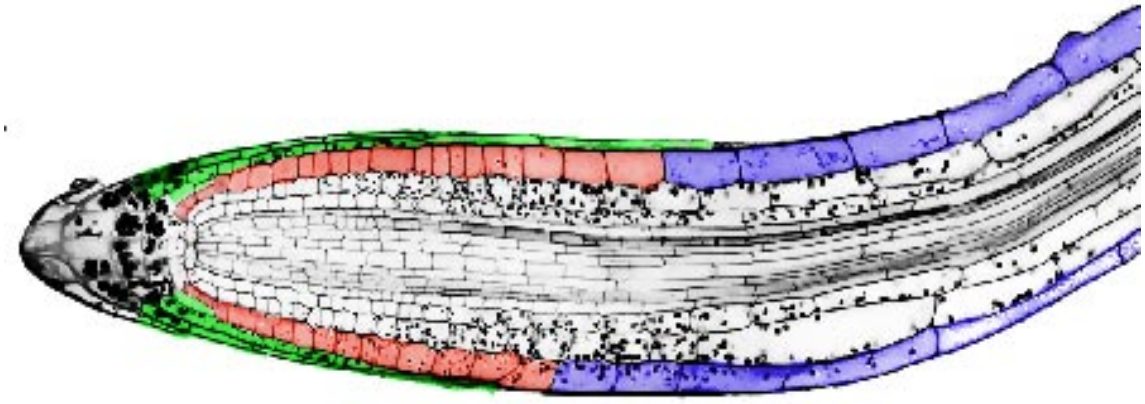






Control of cell division and elongation in the root

The main objective of our work is to better understand the way positional information is exchanged between cells during plant development. We are using the *Arabidopsis* root meristem as a model system and have developed several new approaches to the subject. These include the use of new imaging techniques to visualise individual cells inside intact meristems and new genetic tools for targeted manipulation of cells. In our current work, we are applying these techniques to the characterisation of a crucial cellular interaction that appears to be required for the control of cell division and elongation in the root meristem.

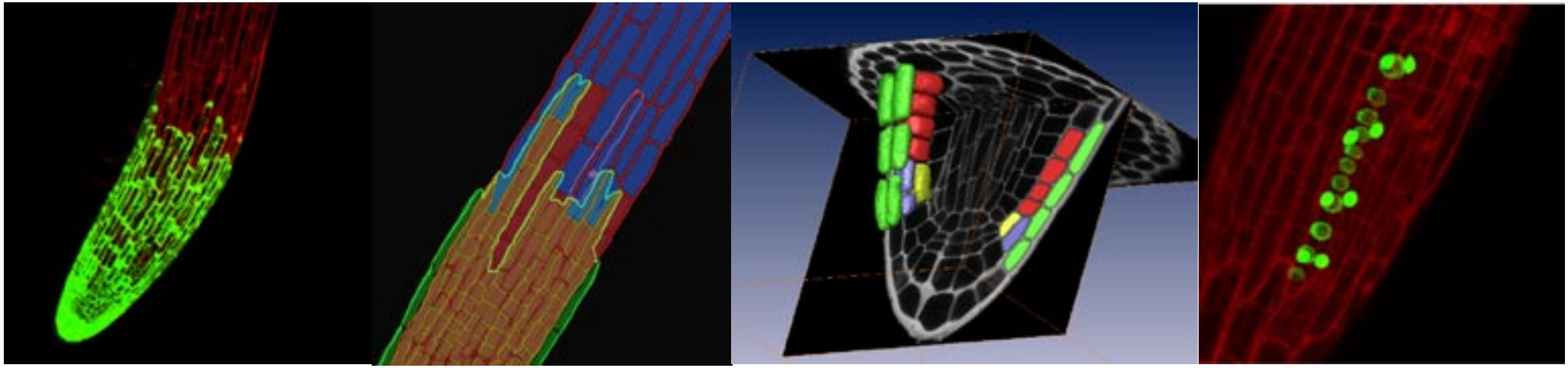


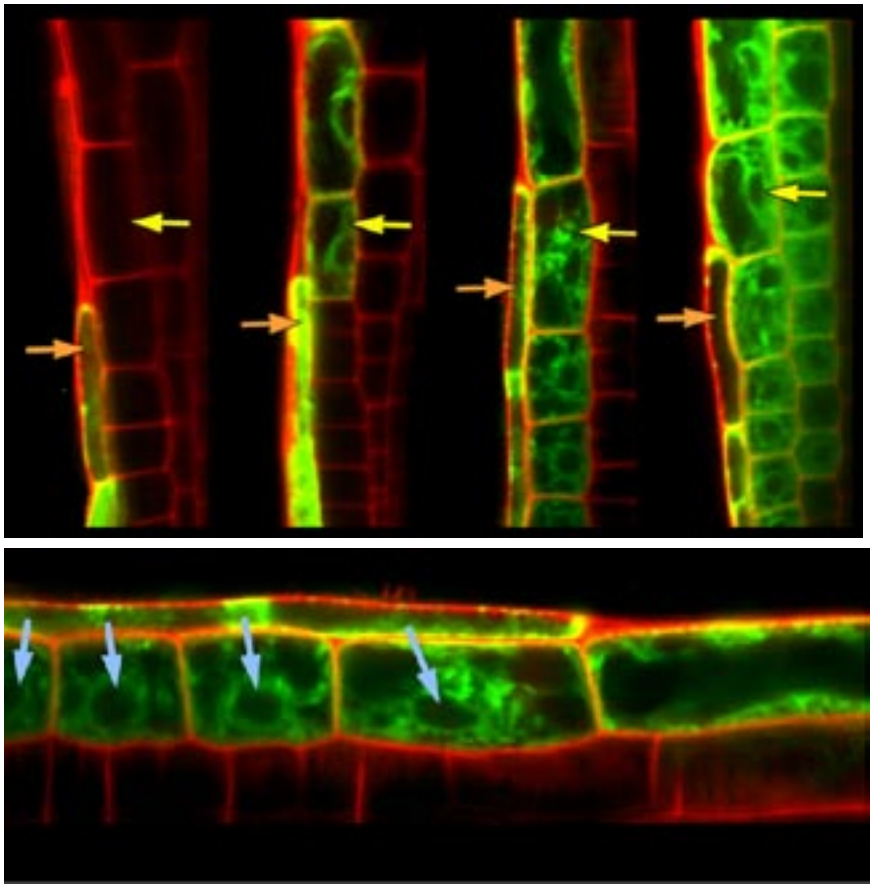
Cell division and elongation in the root meristem.

There is a conserved arrangement of cells within different layers of the lateral root cap and epidermis. The lateral root cap is laid down by division of cells within the meristem, and this forms a short layer on the outside of the root tip as the oldest of the cells eventually undergo programmed cell death. The epidermis lies immediately underneath this layer. At the tip, epidermal cells undergo a rapid set of 4 or 5 divisions, followed by the rapid onset of cell expansion. We have found that, in *Arabidopis*, the boundary between dividing and elongating cells in the epidermis is sharp, and always lies adjacent to the terminal root cap cell in the next layer. In addition, we have a number of enhancer trap lines that clearly mark this boundary.

Mapping cell division in the root meristem.

In order to confirm the location of dividing cells in the root meristem, we have obtained plants expressing a cyclinB1::GFP gene fusion from Dr. Peter Doerner, University of Edinburgh. The marker gene is only expressed in cells undergoing mitosis in these plants, at the G2-M phase transition during the cell cycle. Sarah Hodge has used cyclinB1:GFP fluorescence to map patterns of mitotic activity within the epidermis of intact Arabidopsis root tips. Expression of the marker gene is only found in cells that are contacting the lateral





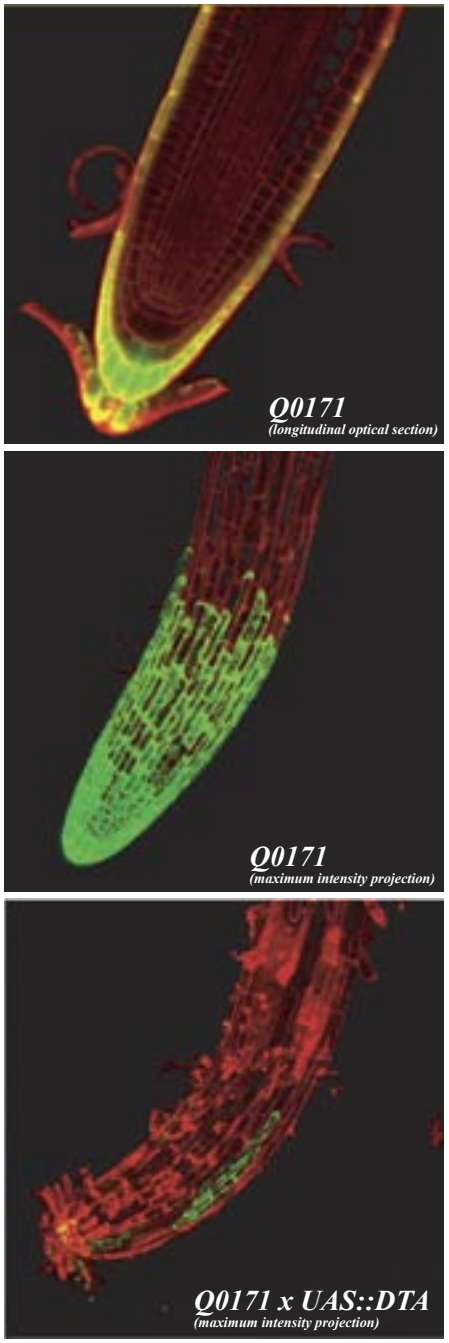
root cap.

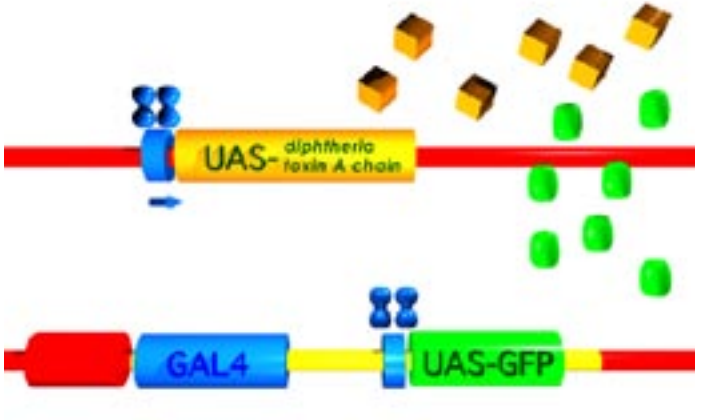
Lineage in the root meristem.

We have generated transgenic *Arabidopsis* lines that contain a histone2b:yellow fluorescent protein fusion, which allows the direct observation of nuclear dynamics in living tissues. We are using this marker to describe the pattern of cell divisions around the epidermis – lateral root cap boundary. In addition, we have used this nuclear marker to construct a system for marking clonal sectors – to allow us to precisely map cell lineages in the root meristem. We have precisely mapped the common origin of lateral root cap and epidermal cells. Cyclin and histone GFP markers provide both dynamic and historical records of cell division in the meristem, and are a valuable complement to 3D segmentation for model building.

Genetic ablation of lateral root cap cells.

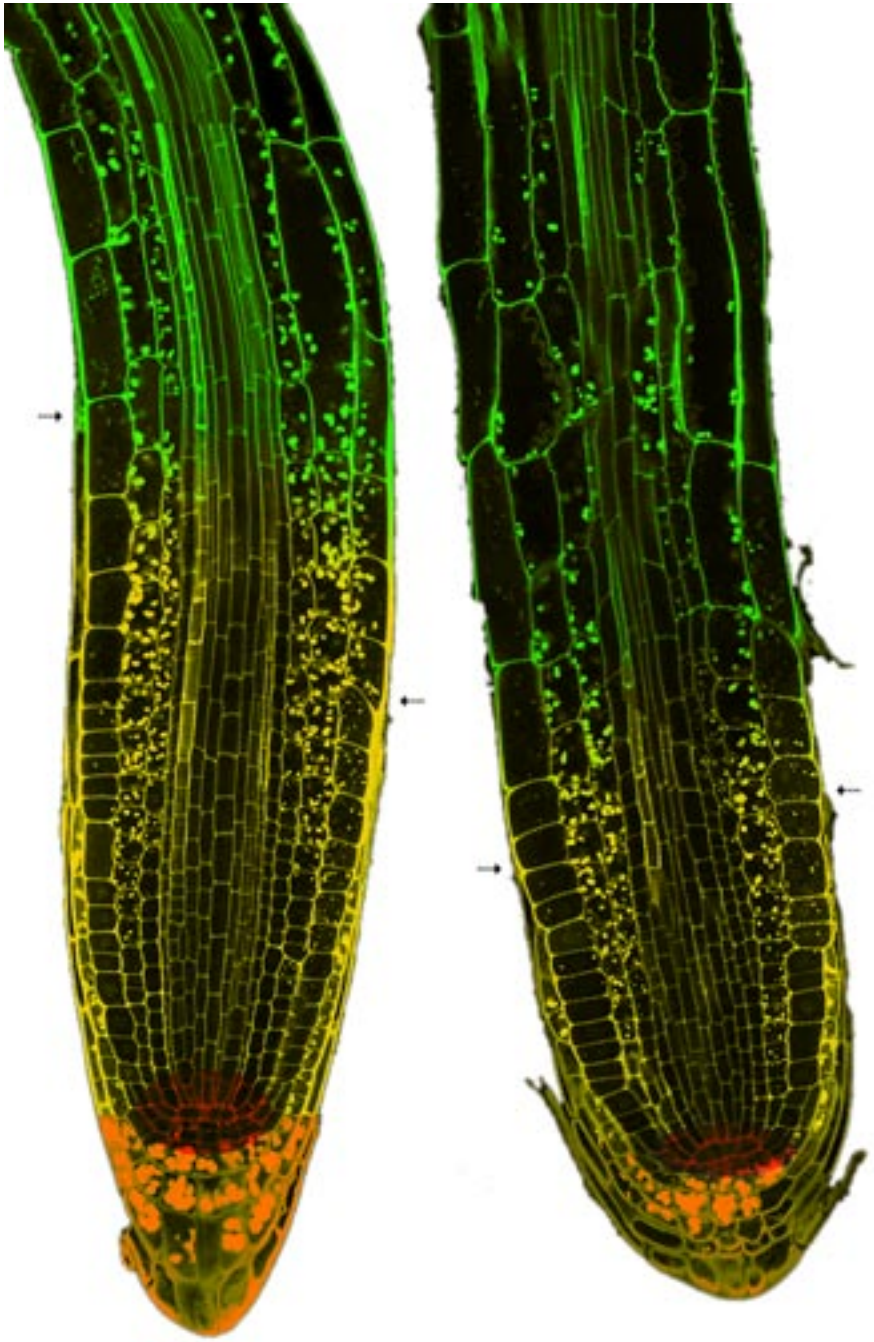
The terminus of the lateral root cap overlies the transition between the cell division and elongation zones in the epidermis. (i) The lateral root cap encases the *Arabidopsis* root tip, as shown in the Q0171 GAL4-GFP





enhancer trap line (top left). (ii) The end of the lateral root cap (green) marks a sharp transition between cell division (red) and elongation (blue) in the underlying epidermis. (iii) Enhancer trap lines show changes in gene expression that mirror this transition. (iv) Lateral root cap (green), small epidermal cells (red) and elongating epidermis (blue) form a conserved cell arrangement (lower panel).

Figure 4. Cell division occurs only under the lateral root cap. Expression of the cyclinB1::GFP gene fusion is a reliable marker for mitosis. In the root epidermis, marker gene expression is only found in cells that are contacting the lateral root cap. A confocal projection (left) and

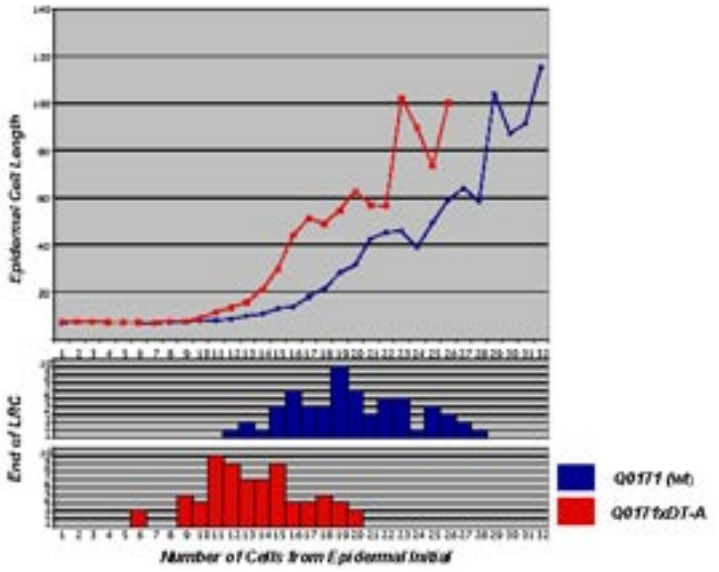


We are now using GAL4 targeted gene expression to kill cells and to trigger ectopic cell divisions in the lateral root cap and epidermal layers of the meristem. We have characterized the epidermis and root cap expression patterns of a number of GAL4-GFP *Arabidopsis* lines and used these to trigger cell death or ectopic cell division in the different layers. We see phenotypes due to the localized death or proliferation of cells which are consistent with the intercellular control of cell division and elongation.

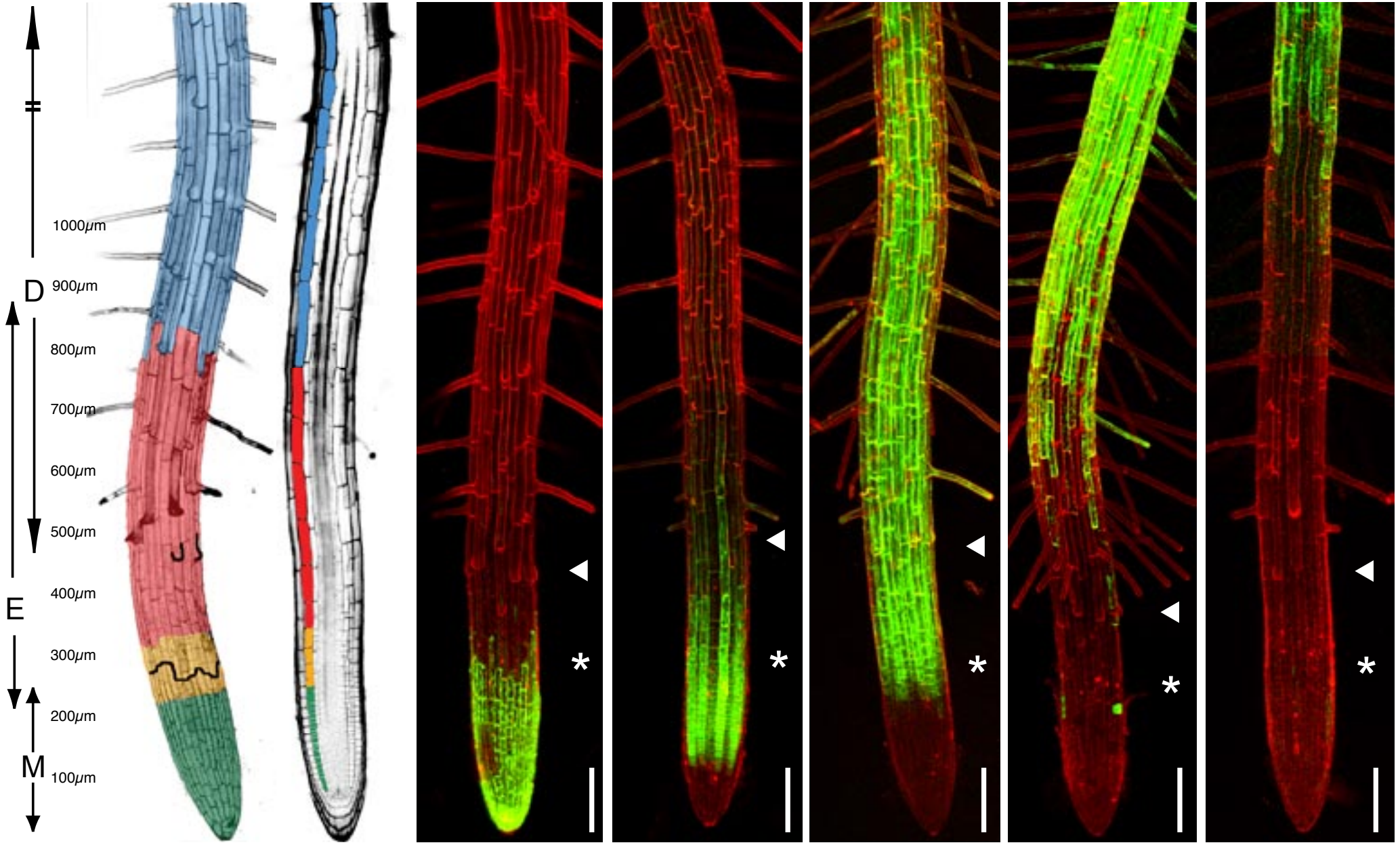
Using an *Arabidopis* line with lateral root cap expression of GAL4, it has been possible to target the diphtheria toxin A chain to these cells, to specifically kill them.

Targeted cell ablation of the lateral root cap. The enhancer trap line Q0171 expresses GAL4 and GFP in the lateral root cap and outer layer of the columella (top left). When this line is crossed to plants containing a diphtheria toxin A chain gene under the control of a GAL4 promoter, root growth is inhibited (top right) due to the targeted ablation of lateral root cap and outer columella cells (bottom left). Killing the lateral root cap cells results in shortening of the division zone and causes epidermal cell elongation to occur closer to the root tip (bottom right). The boundary between the terminus of lateral root cap and the transistion between cell division and elongation in the underlying epidermal layer is conserved.

When the lateral root cap cells are ablated, epidermal cells undergo accelerated differentiation: cell expansion and the formation of trichoblasts are seen immediately adjacent to the root tip. This indicates that lateral root cap cells play a direct role in maintaining the properties of the rapidly dividing epidermal cells that they contact, and suggests that an interaction between two layers is required for the coordination of cell division and root growth.



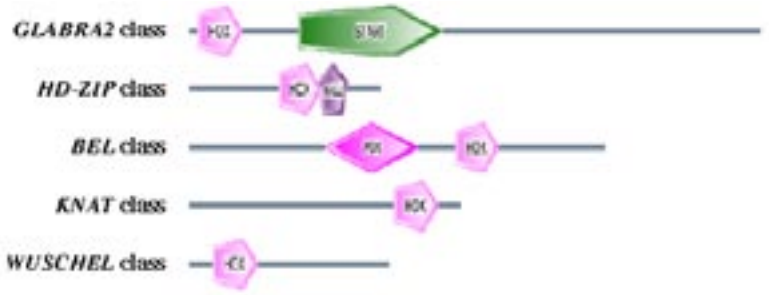
Zonation in the Arabidopsis root meristem.

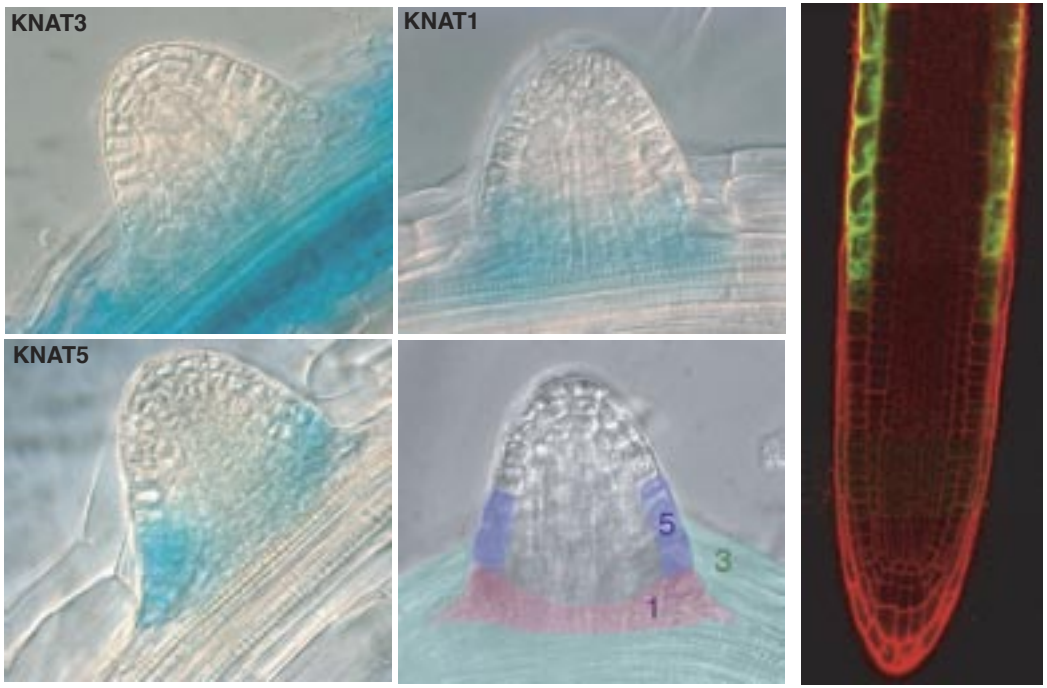


Expression of homeodomain proteins and zonation in the Arabidopsis root meristem.

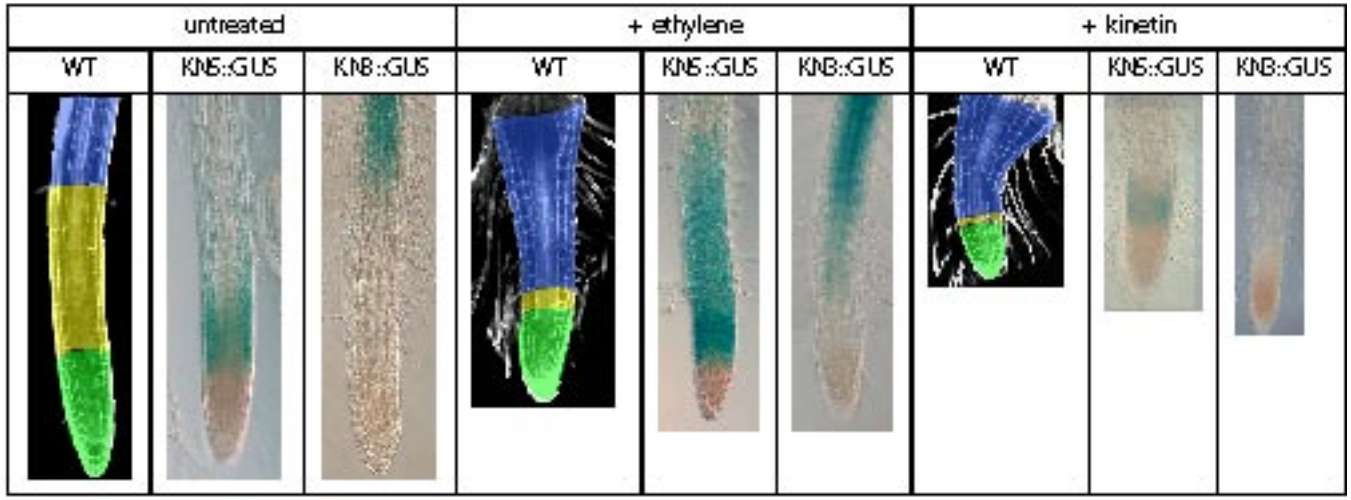
There is a conserved longitudinal arrangement of cells within different layers of the root meristem. A clear example is found in the epidermis and lateral root cap layers at the root tip. The boundary between dividing and elongating cells in the epidermis is sharp, and always lies adjacent to the terminal root cap cell in the next layer. Meristems grow indeterminately and their maintenance requires the precise balance of the cell proliferation and differentiation processes. We are examining the potential role that different classses of homeodomain proteins may play in meristem zonation and maintenance.

Homeodomains were first identified in *Drosophila*, in genes where mutation resulted in homeotic defects. The motif is found in proteins across the animal, plant and fungal kingdoms, and corresponds to a conserved Helix-Loop-Helix-Turn-Helix DNA binding domain. There are 100 homeodomain containing proteins encoded within the *Arabidopsis* genome. These form a highly diverse family, including the KNAT, BEL and HD-ZIP classes of transcription factor. Only a small proportion of these are characterised. However the family is known to contain a number of important gene regulators. For example, members of the KNAT class of homeodomain proteins (KNAT1, KNAT2, STM) are required for shoot meristem initiation and maintenance. Misexpression of KNAT1 or KNAT2 results in ectopic proliferation of shoot meristems, and we have recently demonstrated a role for KNAT genes in growth of the root meristem (E. Truernit, JH, unpublished). In addition, other homeodomain proteins are required for the proper specification of cell fates in *Arabidopsis*. For example, the GLABRA 2 gene (GL2) is required for epidermal patterning in the root.





There are 93 proteins containing homeodomain motifs in the *Arabidopsis* genome and these fall into five classes. A number of these proteins are known to play important roles in integrating cell activities during development. However, most of these putative transcription regulators are uncharacterized. The proteins are known to interact to form ternary complexes in animals and plants. For example, the BEL and KNAT classes of Arabidopsis TALE homeodomain proteins have been shown to physically associate (Bellaoui *et al*., Plant Cell 13: 2455-70). An N-terminal SKY domain and a region just downstream of the homeodomain of BEL1 protein interacts with the MEINOX domains of STM, KNAT1, KNAT2 and KNAT5. This parallels well characterised N-terminal associations between MEIS and PBC homeodomain proteins in animals (Knoepfler *et al*., PNAS 94: 14553-8). Homeodomain protein dimerisation for transcription regulation appears to be a common feature across animals and plants. Different classes of homeodomain genes in Arabidopsis possess different overlapping patterns of expression. Heterodimerisation between homeo-

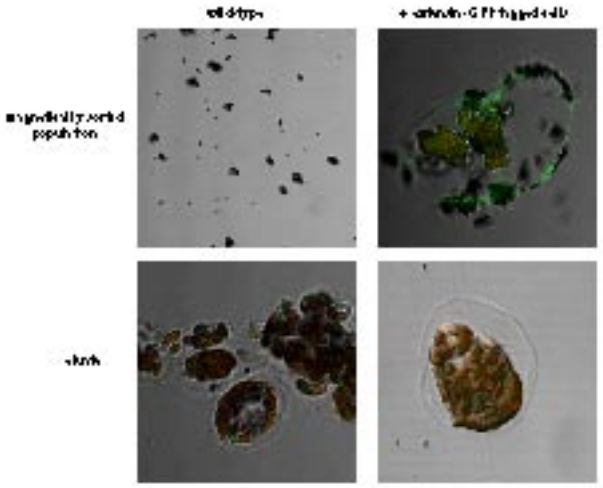


proteins would allow combinatorial control of transcription regulation and the balance of competing states of gene expression during development.

Further, different classes of homeodomain genes in *Arabidopsis* possess different overlapping patterns of expression and appear to respond to different cues. Therefore heterodimerisation would allow combinatorial control of transcription regulation and the balance of competing states of gene expression during development, which is a crucial aspect of meristem maintenance. For example, WUSCHEL promotes stem cell activity within the shoot meristem, while in a complementary fashion, SHOOTMERISTEMLESS acts to prevent differentiation. GLABRA2 is required for proper specification of epidermal cell fates. Members of the HD-ZIP family appear to be involved in plant responses to the environment and KNAT gene expression is regulated by different hormones. Functional interactions between the different homeodomain proteins would provide a simple mechanism for the integration of signals during meristem growth. While a number of the 93 homeodomain genes have been shown to play a role in controlling meristem development, the majority remain uncharacterised. We are attempting to build a more complete map of the gene expression and protein interactions that underly homeodomain protein function in the root meristem.

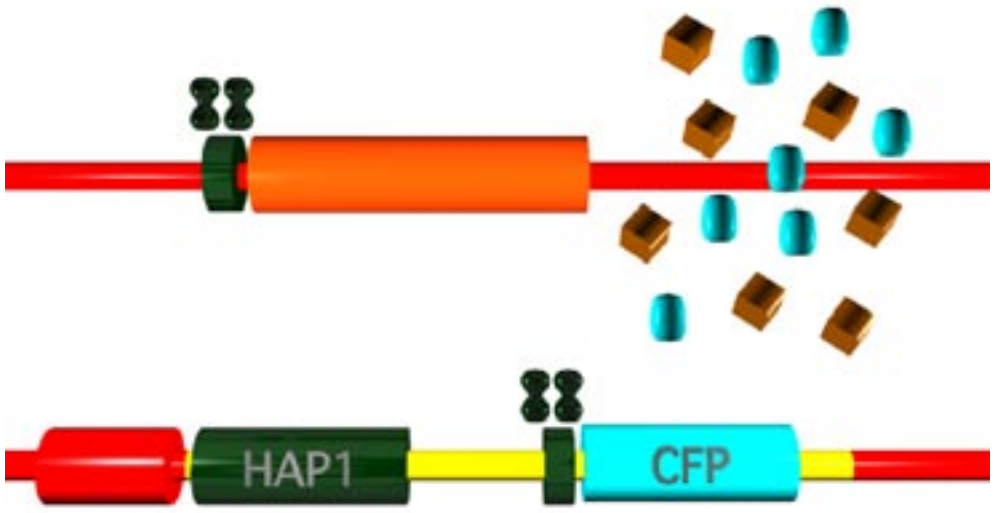
Mapping homeodomain gene expression in isolated cells of the Arabidopsis root meristem.

We are using enhancer trap lines with precisely localized patterns of GFP expression in the root meristem for rapid separation and isolation of marked cells from intact tissue, and for magnetic antibody sorting of the GFP expressing cells. We wish to use these techniques to obtain pure populations of cells from tagged root tissues. The sorted cells would provide a bulk source of chosen cell types, and could be used for the analysis of gene and protein expression in individual cell types. We have developed custom microarrays to identify homeodomain proteins that play a role in root meristem development, and are mapping the transcriptional activity of homeodomain proteins in different zones of the root meristem. We have found evidence for specific hormonal regulation of these homeodomain proteins in the root. For example, the application of either cytokinin or ethylene produces similar shortened root phenotypes, and the conventional view is that cytokinin effects are mediated by elevated ethylene production. However, KNAT3 and KNAT5 promoters respond in opposite ways to exogenous cytokinin and ethylene, suggesting that the phenotype is more likely due to an imbalance of competing processes across the meristem.



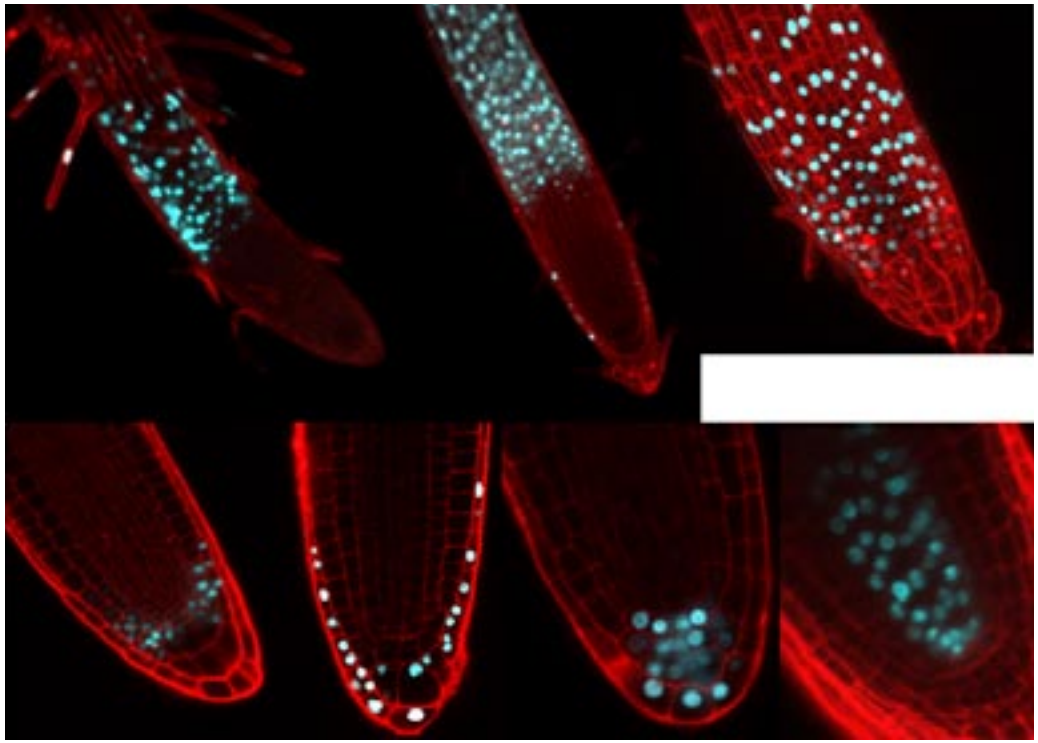
HAP1-based system for gene expression.

The successful application of the GAL4 system has driven the need for additional *GAL4-independent* systems for gene activation and fluorescent cell markers. Additional systems would allow the independent activation of different genes and the simultaneous tracking of multiple cell types. To this end, we have taken another gene from yeast which is a transcription factor closely related to GAL4, named HAP1. The yeast GAL4 protein is a member of a family of zinc-finger (Cys4) transcription factors which are limited to fungi, and homologues have not been found in plants to date. In order to generate a GAL4-independent system for targeted gene expression and generation of cell markers, we have constructed a synthetic HAP1-VP16 gene. HAP1 is another yeast zinc-finger transcription factor related to GAL4, but with a different binding specificity. Yeast genes have a high A/T content and are often poorly expressed in Arabidopsis due to aberrant post-transcriptional processing. Therefore we have constructed a synthetic gene which has an elevated G/C content, and in which the DNA binding domain is fused to the highly active and G/C-rich transcription activator domain of VP16. We have also synthesised an optimised multimeric binding site for HAP1, and cloned this behind a histone2b-CFP reporter (Marion Bauch & J.H., unpublished results).



These elements have been used for the construction of an enhancer trap vector, following the methods proven for the GAL4-based vector. We have used the HAP1-CFP enhancer trap vector to generate a new collection of 13,000 HAP1-CFP enhancer trap *Arabidopsis* lines. This has been funded by the US-based biotechnology firm, Ceres Inc. with the shared objective of making these lines publicly available for academic research. As with the GAL4-GFP lines, around 10-20% of the lines show some expression of the CFP marker gene. Many patterns are bright and some are exquisitely restricted to particular cell types

The highly specific patterns of GAL4 and HAP1 expression seen with the enhancer trap lines allow targeted gene activation. In addition, they provide precise cell fate markers. For example, our *Arabidopsis* line J2301 contains brightly marked root cap and atrichoblast cells. The line has been used to as a cell fate marker during epidermal differentiation in wild-type (Berger *et al*., Current Biology 8:421-430, 1998) and mutant backgrounds (Cnops *et*

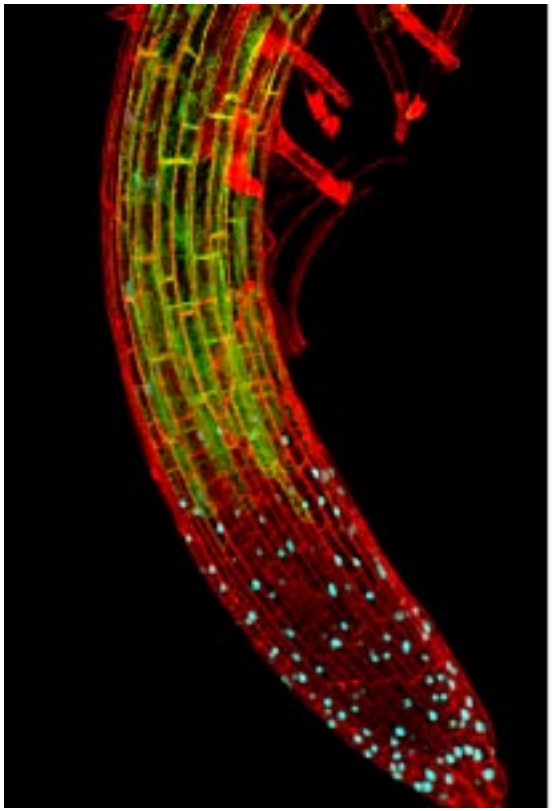


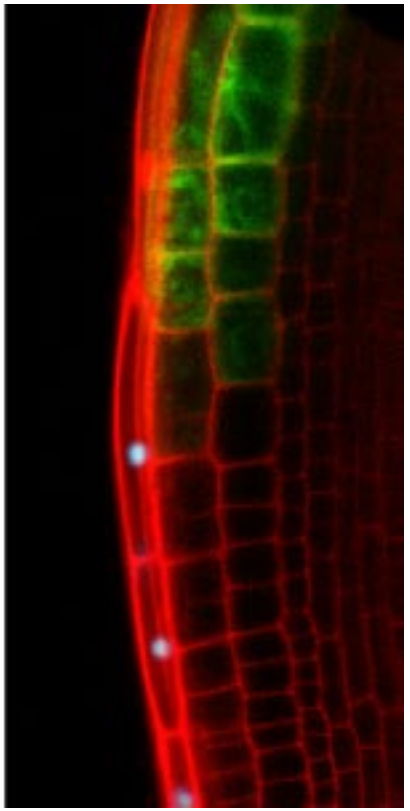
al., Development 127:3385-3394, 2000). The line J2341 contains brightly marked central zone and initial cells in root, and this has been used extensively in studies of auxin transport in the root meristem (Sabatini *et al*., Cell 99:463-472, 2000). Fred Berger has used a number of GAL4-GFP markers for visualising endosperm development, and for evaluation of mutant phenotypes (Boisnard-Lorig *et al*., Plant Cell 13: 495-509, 2002). Many additional studies are ongoing.

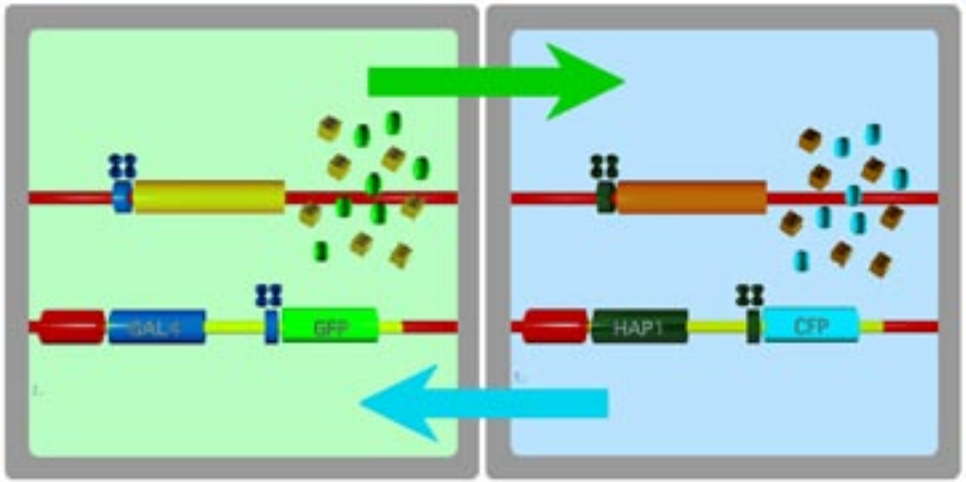
Feedback regulated gene expression.

We can reprogram gene expression within a particular cell type using the GAL4 system. This often produces alterations in neighbouring cells and we need markers to follow the fates of adjacent cell types within the root meristem. Until recently, our best markers were the GAL4-GFP lines, however, we couldn't use two GAL4 patterns together because of crosstalk. The availability of the HAP1 lines means that we can design experiments where GAL4 is used to drive ectopic expression of a cell regulator or toxin, and HAP1 is used to independently report non-cell autonomous perturbations. For example, the figure on the right shows a root meristem with lateral root cap cells marked by expression of a HAP1-CFP enhancer trap and elongating epidermis and cortex cells marked by GAL4-GFP expression.

We now have two independent systems for driving gene expression in the root meristem, and wish to use these as a basis for establishing feedback regulated patterning in *Arabidopsis*. GAL4 and HAP1 enhancer trap vectors can be regarded as a means of obtaining outputs from existing gene regulatory pathways. They can be used to tap into the activity of a particular cellular enhancer and to drive the coordinate expression of any chosen set of genes. GAL4 or HAP1 targeted misexpression may have an effect on the activity of the enhancer that drives its own expression, but this type of feedback is generally unplanned. We wish to deliberately engineer feedback between the two systems and to construct a synthetic system for patterning based on a reaction-diffusion mechanism. Expression of the foreign transcription activators will be used to trigger autoactivation of gene expression and production of diffusible repressors. In this way we plan to trigger the formation of new self-organising patterns that could be used to reprogram plant development.

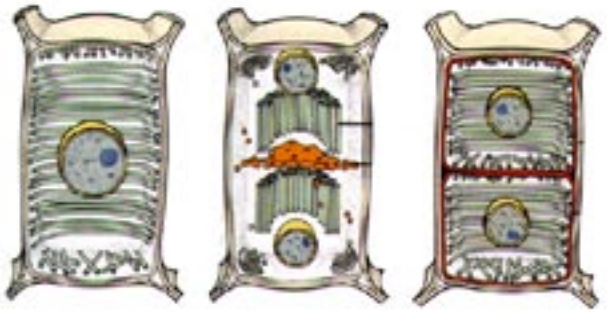




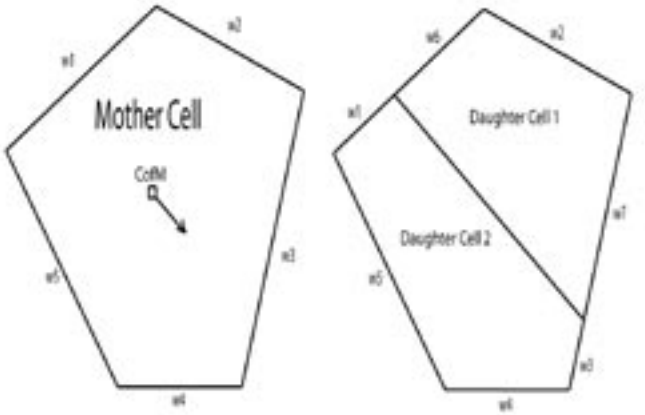


Modelling morphogenesis in plants

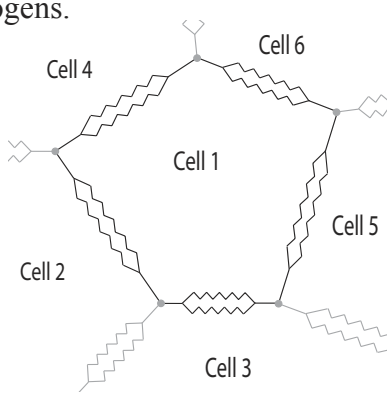
We are experimenting with a dynamic computational system for modelling the cellular morphogenesis of plants. This is based on a self-reproducing cellular automaton which has a dynamic state represented by a set of parameters. The cell-states are determined by a genetic script and a chemical signalling model. The signalling model is an arbitrary set of reacting-diffusing morphogens, the concentrations of which partly define the cell-states. The genetic script is a sequence of logical operations which is repeatedly applied to the cell-state parameters, including the morphogen concentrations. Each cell runs an identical script directly mimicking real genetic logic.

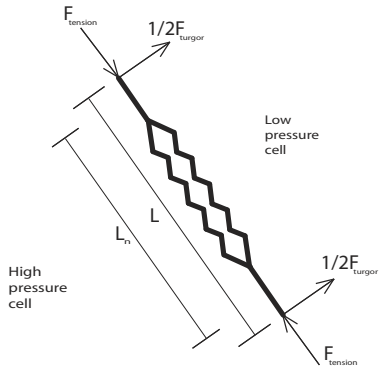


Deposition of new cell walls during plant cell division.



The cells are linked to a spatial/mechanical model that updates the shapes and arrangements of cells in response to their changing states. This provides feedback for the signalling system by affecting the distributions and movement of morphogens.





Spatial model.

The spatial model is two-dimensional with cells restricted to a single plane. Each cell has a closed boundary described by a set of linear elements or *walls*. Each wall forms the interface between two cells or between a cell and the external environment. The geometry of the walls is described by a set of *vertices*, the points where walls intersect. The ultimate output of the spatial model is the position of these vertices. Walls are modelled as a set of springs, one for each adjacent cell, attached to two vertices.

Each spring has fixed stiffness. The natural length of each spring is determined by the state parameters of the appropriate cell. Cell growth is achieved by increasing the natural length of the springs associated with the growing cell. Each cell also has a turgor pressure which exerts a force perpendicular to each wall in an outward direction with respect to the cell. For each time-step of the model the following procedure is executed:

1. Execute genetic script for each cell
2. Adjust mechanical properties of springs (see section 4)
3. Iterate spring model to equilibrium:
   - i. Calculate turgor force for each wall as $(P^{cell1} - P^{cell2}) \times L$
   - ii. Calculate force on each vertex as $F_{(t)} = \sum_{walls} \left(F_{tension} + \frac{1}{2} F_{turgor} \right)$
   - iii. Calculate velocity of each vertex as $v_{(t)} = \left(F_{(t)} - drag \times v_{(t-1)} \right) \times dt$
   - iv. Update vertex positions $p_{(t)} += v_{(t)} \times dt$
   - v. Repeat from (i) until RMS($v_{(t)}$) is small

Cell Growth.

Each cell has three parameters associated with its growth: growth rate, growth axis, and anisotropy. The driving force for cell growth is turgor pressure. This causes the walls (springs) to be in tension and have an associated extension. In this state the cell cannot easily expand due to the stiffness of the springs forming its walls. By resetting the natural length of the cell's springs, we can simulate structural changes in the cell wall that relieve the tension and allow further growth. In this way pressure drives expansion by stretching the walls and the cell responds by restructuring the walls to allow more extension.

Cell Division.

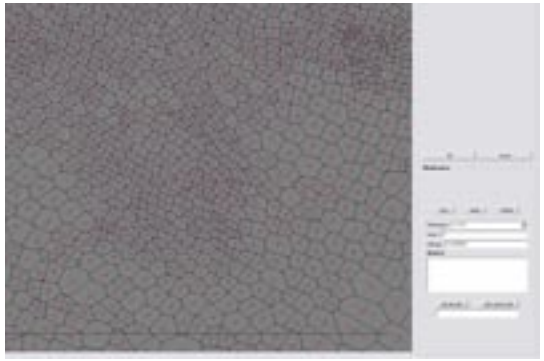
Cell division is instigated by the genetic script of the dividing cell. All divisions are currently approximately symmetric. The dividing wall is inserted parallel to the division orientation vector and passing through the centre of mass of the cell. Any existing walls forming the boundary of the dividing cell that are intersected by the dividing wall are split into two halves. The resulting set of walls is allocated to two new cells either side of the dividing wall.

Genetic Script.

The genetic script is implemented via an embedded Tcl system. It may perform any sequence of Tcl instructions (e.g. logical conditions, resetting) on the above mentioned cell parameters. A list of cell parameters is shown on the left hand side of this panel. In addition two procedures are defined. The *divide* procedure instigates cell division, instructing the spatial model to adjust itself accordingly. The script may also cause the cell to die via a *kill* procedure.

Morphogens.

The signalling model is designed to be sufficiently broad to incorporate a range of biological mechanisms while remaining a coherent system. It is based on reacting and diffusing *morphogens*, first proposed by Turing as a mechanism for biological pattern formation. This allows for any system of chemical reactions coupled with passive transport modelled by the diffusion equation. Diffusion allows us to model long-range (high diffusion rate), short-range cell-to-cell (low diffusion rate), and cell-autonomous (zero diffusion rate) signal molecules within the same mathematical framework.



Algorithm.

The model is computed in a discrete time scheme as follows:

1. Execute genetic script for each cell
2. Iterate the reaction-diffusion system over N_s sub-time-steps
3. Adjust the mechanical properties of the walls
4. Solve the mechanical spring model
5. Repeat from step 1.

Cellular parameters under genetic control:

Growth rate: *The relative rate of growth of the cell defined on [0,1].*

Anisotropy: *The degree of polarity in cell growth ranging from zero (isotropic growth) to one (growth restricted to direction of polarity)*

Growth axis: *The direction along which anisotropic growth will occur most. This is defined relative to the dividing wall which created the cell, and may be parallel or perpendicular to it.*

Division axis: *The direction along which the dividing wall is formed during mitosis. Again this is defined relative to the dividing wall which created the cell.*

Turgor: *The internal turgor of a cell is modelled to allow it to maintain its shape and to allow the transmission of hydrostatic pressure across the tissue.*

Morphogen production rates (pu0, pu1, … pun): *The rate at which each cell is producing or degrading each of the morphogens in the system.*

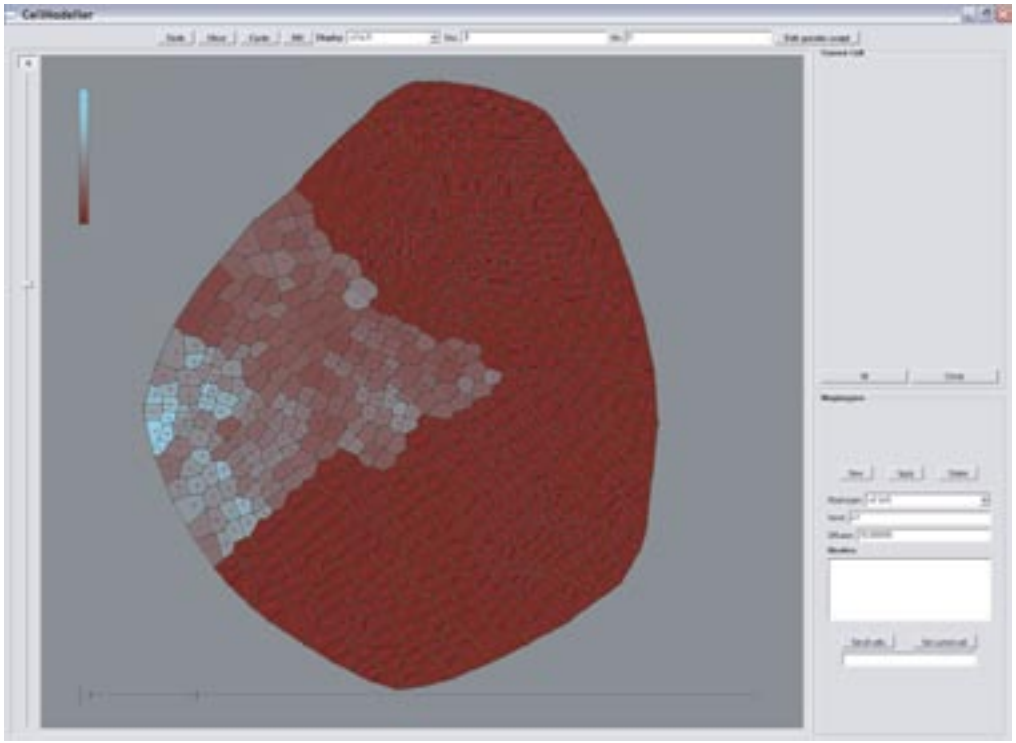
Cell Status:

Timer: *A timer variable is initialised to zero when a cell divides: analogous to the cell cycle.*

Volume/Size: *In 2D this is the area enclosed by the cell boundary. This may not be directly changed by the script but is affected by altering the growth parameters.*

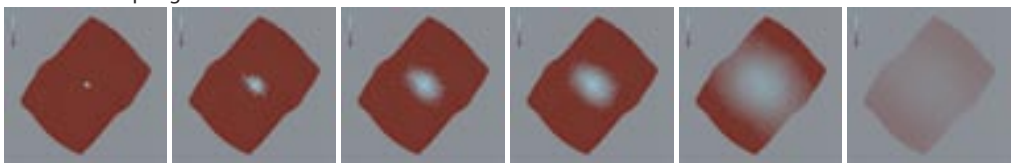
Morphogen concentrations (u0, u1, …un): *These form the basis of the chemical patterns which may then influence cell behaviour via the genetic script. This is analogous to gene expression in response to signalling molecules.*

CellModeller software

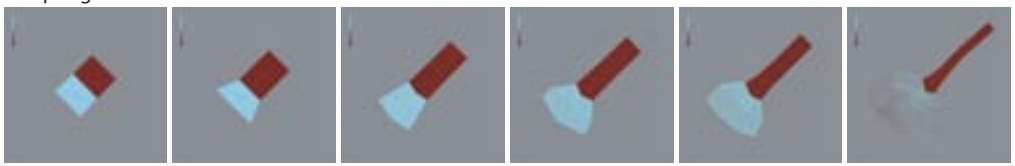


cell division and elongation

diffusible morphogens



morphogenesis

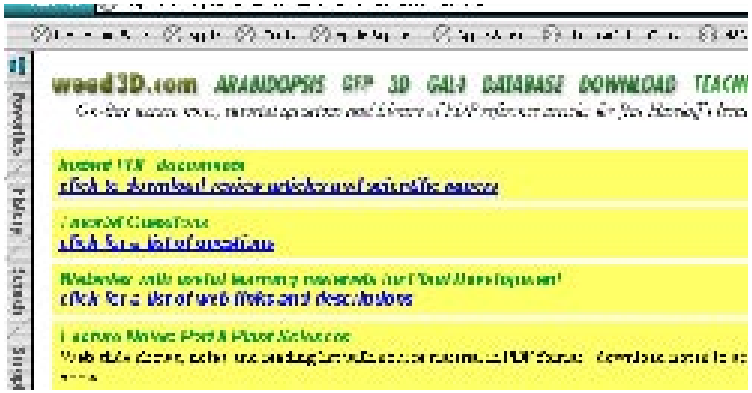


Implementation of the cell modelling software.

The cell modelling system has been implemented as a standalone program, CellModeller, written in C++. The program has been compiled for the Windows platform, and we hope to provide an OSX version to improve access for biologists. The program provides an OpenGL based window for visualisation of cell parameters and dynamic behaviour, along with graphical interfaces for control of the model. We have taken some care to base the model on algorithms that have a direct physical or genetic basis in plants. For example, our double spring model for plant cell walls is based upon the dual activities of adjacent cells during growth, and the model allows for the role of turgor in driving cell expansion, and allows for anisotropic expansion and cell division. These are key processes in plant development and the parameters are directly accessible within the dynamic model. Accordingly, the program interface is also designed with the biologist and the experimental system in mind. We have included an interface for programming the 'genetic' behaviour of the cells using Tcl scripts. In this way, morphogen gradients can be used to establish and maintain patterns, and cells may be programmed to differentiate accordingly. This allows one to model the genetics of existing cellular interactions. In addition, we have made provision for the direct interaction with chosen cells during the execution of the model, to mimic perturbations that might be introduced during a cell biology experiment.

The program has proved robust and the models show lifelike behaviour. We are beginning to test the program, side-by-side with genetic experiments using foreign transcription activators and different coloured fluorescent proteins, to experiment with artificial pattern formation in *Arabidopsis* tissues. We are also planning to introduce additional cell parameters into the modelling system that would reflect the known polarity of plant cells. This would allow for easy incorporation of asymmetrically distributed elements that are involved in transport of information between cells, and are presumably part of feedback loops. Examples of these are the asymmetrically localised PIN gene products required for initiation and maintenance of auxin-mediated cell polarity. Information and compiled code is available at our web site: http://www.plantsci.cam.ac.uk/Haseloff

Undergraduate lecture materials.



I give second and third year lectures at the University of Cambridge in the area of plant development and biotechnology. An electronic version of the lecture slides, notes, and additional teaching material can be found on our web site: http://www.plantsci.cam.ac.uk/Haseloff (click the "teaching" menu choice to navigate to the relevant section). The electronic version of the lecture notes contains links to the recommended reading texts, which can be downloaded in PDF format, and viewed directly in a web browser. A number of links to original scientific articles and an index of advanced references are also provided, and the teaching section of the web site also contains tutorial questions and *Arabidopsis*-related web links.

Low cost fluorescence techniques for teaching.

Over the last decade, the use of autofluorescent proteins has revolutionised biological research. The ease of use of these fluorescent markers would make them especially suitable for use in teaching. Yet most undergraduate practical teaching in plant sciences does not make significant use of fluorescent proteins and most students are aware of them only through images presented in lectures. Hence there is a growing gap between practices in research laboratories and the methods used by undergraduates in their courses. This is largely because of the lack of cheap and robust confocal microscopes suitable for undergraduate use. While a high quality transmitted light microscope can be purchased for around £1000 in the UK, the cheapest fluorescence microscope will cost at least 5 times that amount.

Taking advantage of recent developments in LED technology, optical filters and cameras I have constructed prototype instruments for fluorescence microscopy and detection that are much cheaper than the commercially available alternatives. For example, suitable beamsplitters can be constructed from low cost dichroic material used for theatrical lighting, and a 5W blue LED can provide the basis for a fluorescence illuminator for around £20. In addition, I am adapting low cost fibre optics and illuminators used for domestic and pool lighting for fluorescence detection. While these might appear rather low key activities for a research lab, the availability of cheap fluorescence instrumentation has the potential to fundamentally improve our scientific teaching and recruitment into plant research.

We are developing transgenic *Arabidopsis* lines, fluorescent protein reagents and protocols that can be used for simple teaching practicals.