CHAPTER 9

GFP Variants for Multispectral Imaging of Living Cells

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I. Introduction

Developing multicellular tissues or organs generally demonstrate a capacity for self-organization. For example, wounded tissues can generally respond in a robust and coordinated fashion to allow repair, and local induction events can initiate prolonged and coordinated developmental processes (such as in limb bud formation). These types of developmental plasticity and functional autonomy are particularly evident in plant tissues. The basic features of a plant's body plan are established during embryogenesis; however, its final form results from the continued growth of meristems and the formation of organs throughout its life, often in a modular and indeterminate fashion. Plant cells are constrained by rigid cell walls and are generally nonmotile, so there is the clear possibility that cell fates within a meristem are determined by lineage. However, evidence from plant chimera and wounding studies have demonstrated a more important role for cell–cell interactions during fate determination (reviewed in Steeves and Sussex, 1989). It is likely that positional information during plant development

is obtained via cell–cell contact and that the coordination and fate of cells within a developing meristem may be determined by a network of local cellular interactions. It is also likely that intercellular communication plays a coordinating role in the development of all multicellular organisms. We are using the *Arabidopsis thaliana* root meristem as a model system for investigating intercellular interactions. The root meristem possesses indeterminate growth and has a simple and transparent architecture. *Arabidopsis* is amenable to genetic manipulation, and one can routinely generate transgenic lines for work with the intact organism. To dissect local cell–cell interactions it is crucial that we be able to (*a*) clearly image individual cells inside living meristems and (*b*) have the means to perturb them.

Therefore, we have adapted the jellyfish green fluorescent protein (GFP) for use as a directly visible gene marker in *Arabidopsis* and have developed genetic and optical techniques that allow us to visualize and manipulate cells within living plants.

II. Green Fluorescent Protein Markers

Marker genes have proven extremely useful for reporting gene expression in transformed cells, and the β-glucuronidase (GUS) gene (Jefferson *et al.*, 1987) has been used extensively in transgenic plants. Transformed tissues or patterns of gene expression can be identified histochemically, but this is generally a destructive test and is not suitable for assaying primary transformants, for following the time course of gene expression in living plants, or as a means of rapidly screening segregating populations of seedlings. The GFP from the cnidarian jellyfish *Aequorea victoria* shares none of these problems, and there has been much interest in using the protein as a genetic marker in transgenic *Arabidopsis thaliana*.

Aequorea victoria are brightly luminescent, with glowing points around the margin of the jellyfish umbrella. Light arises from yellow tissue masses that each consist of about 6000–7000 photogenic cells. The cytoplasm of these cells is densely packed with fine granules that contain the components necessary for bioluminescence (Davenport and Nichol 1955; Morin and Hastings 1971). In other bioluminescent coelenterates these have been characterized as 0.2-micron-diameter particles enclosed by a unit membrane, and have been termed lumisomes (Anderson and Cormier 1973). The components required for biolumines-cence include a Ca^{2+}-activated photoprotein, aequorin, that emits blue-green light, and the accessory protein, GFP, which accepts energy from aequorin and reemits it as green light (Morise *et al.*, 1974). GFP is an extremely stable protein of 238 amino acids (Prasher *et al.*, 1992). The fluorescent properties of the protein are unaffected by prolonged treatment with 6 *M* guanidine HCl, 8 *M* urea, or 1% sodium dodecyl sulfate (SDS), and two-day treatment with various proteases such as trypsin, chymotrypsin, papain, subtilisin, thermolysin, and pancreatin at

concentration of up to 1 mg/ml fail to alter the intensity of GFP fluorescence (Bokman and Ward 1981). GFP is stable in neutral buffers up to 65°C, and displays a broad range of pH stability from 5.5 to 12. The protein is intensely fluorescent, with a quantum efficiency of approximately 80% and a molar extinction coefficient of 2.2×10^4 cm^{-1} M^{-1} (Morise *et al.*, 1974) (after correction for the known molecular weight). GFP fluoresces maximally when excited at 400 nm, with a lesser peak at 475 nm, and fluorescence emission peaks at 509 nm.

The intrinsic fluorescence of the protein is due to a unique covalently attached chromophore that is formed posttranslationally within the protein upon cyclization and oxidation of residues 65–67, Ser-Tyr-Gly (Prasher *et al.*, 1992; Cody *et al.*, 1993; Heim *et al.*, 1994). Several genomic and cDNA clones of *gfp* have been obtained from a population of *A. victoria* (Prasher *et al.*, 1992). The *gfp* gene contains at least three introns, and the coding sequence derived from one of the cDNA clones, pGFP10.1 has been used for protein expression, first in *Escherichia coli*, then in *Caenorhabditis elegans* (Chalfie *et al.*, 1994; Heim *et al.*, 1994; Inouye and Tsuji, 1994), and *Drosophila melanogaster* (Wang and Hazelrigg 1994). Fluorescent protein has now been produced in a number of heterologous cell types, and there appears to be little requirement for specific additional factors for posttranslational modification of the protein, which may be autocatalytic or require ubiquitous factors.

The bright intrinsic green fluorescence of GFP allows it to be directly visualized in transformed cells, and we wished to use the protein as a simple marker for transformation and misexpression studies. However, we found that the wild-type GFP cDNA was not expressed properly in *Arabidopsis*, and we have needed to extensively modify the gene (Haseloff and Amos, 1995; Siemering *et al.*, 1996; Haseloff *et al.*, 1997).

1. *Removal of a cryptic intron.* We discovered that the GFP mRNA sequence is efficiently mis-spliced in transgenic *Arabidopsis* plants, resulting in the removal of 84 nucleotides from within the coding sequence, between residues 380 to 463. We removed the cryptic intron by mutagenesis, allowing proper expression (Haseloff *et al.*, 1997).

2. *Subcellular localization of GFP.* We found that GFP accumulates within the nucleoplasm of plant cells, as it does in other organisms, and that it was difficult to regenerate plants from very brightly transformed tissues. This apparent mild toxicity may be due to the generation of fluorescence-related free radicals during growth under light, which might then lead to DNA damage. We have therefore targeted GFP to different subcellular compartments in transgenic plants and assayed for improved regeneration and fluorescence. One of our constructions, which is excluded from nuclei and is retained within the endoplasmic reticulum in *Arabidopsis*, consistently produces bright and healthy transformants.

3. *Thermotolerant GFP mutants.* We have shown that wild-type GFP is thermosensitive and fluoresces poorly at temperatures above 25°C. We have subjected

our modified GFP coding sequence to PCR-based mutagenesis and have isolated a thermotolerant mutant with improved fluorescence. The mutant contains two altered amino acids (V163A, S175G) that greatly improve folding of the apoprotein at elevated temperatures (Siemering *et al.*, 1996).

4. *Altered spectral properties.* The fluorescence excitation of wild-type GFP peaks at wavelengths of 400 and 475 nm, with the 400-nm peak predominating. We have recombined a published mutant of GFP (I167T; Heim *et al.*, 1994) with our improved mutant and have produced a variant that has dual excitation peaks of almost equal amplitude (Siemering *et al.*, 1996). This allows the efficient use of techniques that require either UV or blue light excitation of the protein, for example when screening GFP-expressing plants with a UV lamp or when using blue laser-light-excited confocal microscopy.

All of these alterations have been 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. These improved mutant genes have also proven useful for studies in animal cells (Zernicka-Goetz *et al.*, 1996; Zernicka-Goetz *et al.*, 1997).

Other groups have been struggling with similar problems in other systems, and a number of functionally similar *gfp* variants are now available. For example, *gfp* variants with "humanized" or other optimized codon usage have been produced for better expression. These also show improved levels of expression in plants (Chiu *et al.*, 1996; Haas *et al.*, 1996; Pang *et al.*, 1996). Although these genes were expected to provide better translation efficiency, it is likely that these alterations also confer some degree of immunity from aberrant RNA processing in plants. A number of workers have obtained GFP mutants that show brighter fluorescence in heterologous cell types, and it is likely that the improved properties result from better folding of the proteins. For example, the V163A mutation has been generated independently by at least three different groups (Crameri *et al.*, 1996; Heim and Tsien 1996; Kohler *et al.*, 1997), and this residue may play a pivotal role in folding of the protein. In addition, Cormack *et al.* (1996) introduced large numbers of random amino acid substitutions into the 20 residues flanking the chromophore of GFP. They used fluorescence-activated cell sorting to select variants that fluoresced 20- to 35-fold more intensely than wild type. They also showed that the folding of these mutant proteins was more efficient during expression in bacteria. One of these variants, GFPmut1 (Cormack *et al.*, 1996), contains two amino acid differences, F64L and S65T, located within the central α-helix of the protein, adjacent to the chromophore. The V163A and S175G mutations that we have isolated are positioned on the outer surface of the protein (Ormo *et al.*, 1996; Yang *et al.*, 1996), and recombination of these two sets of mutations results in an exclusively blue-light-excited GFP with markedly improved fluorescence properties (Zernicka-Goetz *et al.*, 1996, 1997). The beneficial effect of both sets of mutations on protein folding and their apparent additive effect suggest that they may play separate roles in the folding or maturation process.

III. Imaging of Living Cells

GFP expression and localization can be visualized directly. without a prolonged and lethal staining procedure. The expression of GFP within an organism produces an intrinsic fluorescence that can used to "paint" particular cells or cellular processes. The fluorescent properties of the protein allow (*a*) simple screening for gene expression in living plants by inspection with a UV lamp. and (*b*) high-resolution imaging of subcellular events in living cells using fluorescence microscopy.

GFP can possess alternate protonated and anionic forms of its chromophore. which allow excitation with long-wavelength UV (395 nm) or blue light (475 nm). respectively (Cody *et al.*, 1993; Heim *et al.*, 1994). The relative amplitude of the two excitation peaks is predominantly determined by the amino acid sequence and protein environment around the chromophore. rather than the solvent. and mutant forms of GFP have characteristic excitation spectra. Most available forms of GFP have been optimized for excitation at either of these wavelengths: for example GFP variants containing the S65T mutation (Heim *et al.*, 1995) are widely used and can be excited by blue. but not UV. light. In contrast. we have chosen to use GFP derivatives (mGFP5) with more versatile spectral properties for our initial work in *Arabidopsis*. The mGFP5 protein is equally well excited with either long-wavelength UV or blue light. This allows the use of ultraviolet excitation for simple inspection of transformed material. because the illuminating wavelength is poorly detected by the human eye. In addition. transformed plant tissue or seedlings growing in sterile culture can be simply scored for GFP expression using fluorescence microscopy. and we have used the marker to simplify an enhancer-trap screen. Efficient blue-light excitation of GFP also allows work with commonly available microscope filter sets and laser sources. such as those used during confocal microscopy (Fig. 1. see color plate).

Confocal imaging allows precise visualization of fluorescent signals within a narrow plane of focus. with exclusion of out-of-focus blur. and the technique permits the reconstruction of three-dimensional structures from serial optical sections. Intact plant tissue proves a difficult subject for fluorescence microscopy because it consists of deep layers of highly refractile cell walls and aqueous cytosol. and contains various autofluorescent and light scattering components. There are two approaches to the difficulties imposed by these conditions. (*a*) to fix and to clear the tissue with a high-refractive-index mounting medium. or (*b*) to directly image living tissue using suitably corrected microscope optics. In our experience. it has proved difficult to effectively clear *Arabidopsis* wholemounts without causing artifacts or losing GFP fluorescence. and there are considerable advantages to working with living tissues, so we have pursued the second approach. *Arabidopsis* seedlings can simply be mounted in water for microscopy and examined using a long-working-distance water-immersion objective to mini-

mize the effects of spherical aberration when focusing deep into an aqueous sample (Haseloff and Amos, 1995). Even with the use of such a specialized objective (Nikon 60× planapochromat, numerical aperture 1.2, working distance 220 μm), image quality degrades rapidly for optical sections deeper than 60–80 μm within the tissue. However, the small size of *Arabidopsis* seedlings allows very useful imaging despite this limitation and, for example, median longitudinal optical sections can be obtained from intact roots (Fig. 2A, see color plate).

Protocol: Mounting and Observing GFP-expressing *Arabidopsis* Seedlings

A. Growth of *Arabidopsis* in sterile culture.

1. Twenty to one hundred transgenic *Arabidopsis* seeds were placed in a 1.5-ml microfuge tube and washed for about 1 min with 1 ml of ethanol.

2. The seeds were then incubated with 1 ml of a surface-sterilizing solution containing 1% (w/v) sodium hypochlorite and 0.1% (v/v) NP40 detergent for 15 min at room temperature.

3. The seeds were then washed three times with 1 ml of sterile water and transferred by pipette to agar plates containing GM medium (Valvekens *et al.*, 1988):

1× Murashige and Skoog basal medium with Gamborgs B5 vitamins (Sigma)

1% sucrose

0.5 g/liter 2- (*N*-morpholino) ethanesulfonic acid (MES)

0.8% agar (adjusted to pH 5.7 with 1 *M* KOH)

Twenty-five mg/liter kanamycin was added if antibiotic selection of transgenic seedlings was necessary.

These procedures were performed in a laminar flow hood.

Alternatively, for extended time-lapse imaging of roots, sterile seeds were sown in coverslip-based vessels (Nunc) that comprised 4 wells, each containing about 400 μl of low-gelling temperature agarose with GM medium. The roots of these plants grow down through the medium and then along the surface of the coverslip. The roots are then ideally positioned for high-resolution microscopic imaging through the base of the vessel.

4. Sealed plates or vessels were incubated for 1–3 days in the dark at 4°C, and then transferred to an artifically lit growth room at 23°C for germination.

5. *Arabidopsis* seedlings germinate after 3 days and can be used for microscopy for several weeks. Root and shoot tissues can be directly scored for GFP expression using a inverted fluorescence microscope (Leitz DM-IL) fitted with filter sets suitable for UV (Leitz-D; excitation filter 355–425 nm, dichroic mirror 455 nm, longpass emission filter 460 nm) and blue (Leitz-I3; excitation filter 450–490 nm, dichroic mirror 510 nm, longpass emission filter 520 nm) light excitation of GFP. Roots, which grow along the base of the Petri dish can be observed directly by epifluorescence microscopy through the clear plastic base.
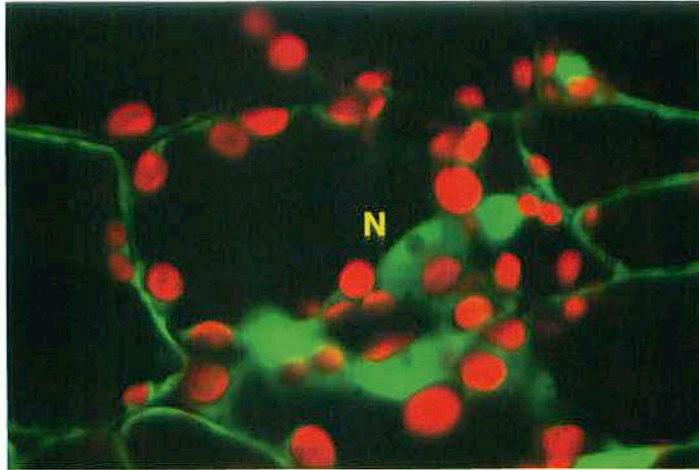
Ch.9, Fig. 1 Confocal optical sectioning of intact *Arabidopsis* plants. An *Arabidopsis* seedling that expressed a 35S promoter-driven GFP gene was subjected to microscopic examination. The image was collected using a Nikon Optiphot microscope equipped with a Biorad MRC-600 confocal scan head, and a Nikon planapo 60× (NA 1.2) water-immersion lens. GFP and chlorophyll were excited using the 488- and 568-nm lines, respectively, of a 25-nm krypton–argon ion laser. The green and red emissions were collected in separate channels and combined and pseudocolored using Adobe Photoshop. Individual cells and subcellular structure can be clearly resolved. Nuclear accumulation of GFP is seen in these cells (N), with exclusion from the nucleolus. The cytoplasm is pressed to the cell wall in these highly vacuolate cells. Chloroplasts are red autofluorescent.
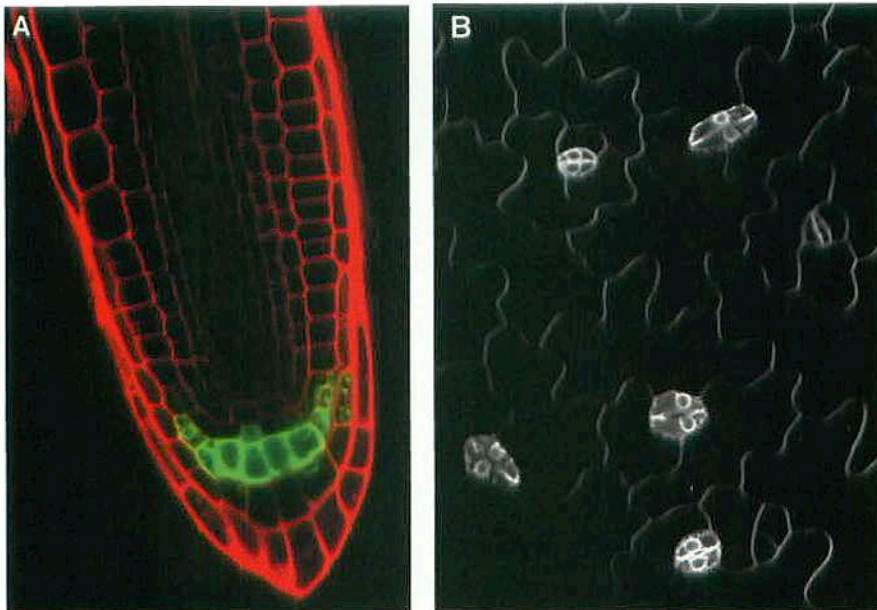
Ch.9, Fig. 2 Vital fluorescent dyes for counterstaining. (A) Transverse longitudinal section of a 5-day-old *Arabidopsis* root tip that has been stained with propidium iodide. Propidium iodide fluorescence is seen in the red channel, and reveals the outlines of cells in the root tip. GFP expression in this *Arabidopsis* transgenic line Q1630 (Sarah Hodge and J. Hasselhoff, unpublished results) is seen in part of the root cap, and is shown in the green channel. (B) Optical section through the epidermal layer of an FM 1-43 stained cotyledon of a 5-day-old seedling (Line J0991; J. Hasselhoff, unpublished results). FM 1-43 selectively stains the plasma membrane, to provide an outline of these cells. GFP fluorescence was collected in the same channel, and can be seen to effectively "fill" guard cells, which express the protein.
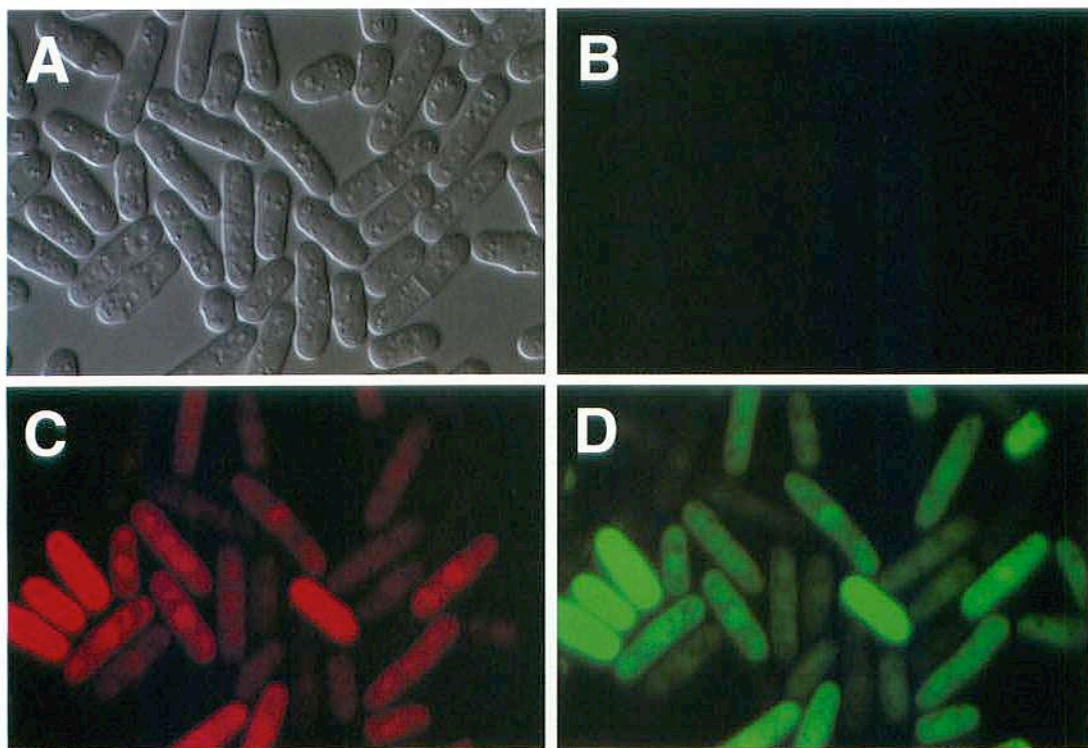
Ch.8, Fig. 5 Photoactivation of GFP. (A) DIC image before photoactivation (0.04-s exposure). (B) The same field in the Texas Red channel, before photoactivation (2-s exposure). (C) The same field in the Texas Red channel after 5-s photoactivation with blue light (2-s exposure). (D) The same field in the fluorescein channel, after photoactivation (0.5- exposure).

Shoot tissues were directly observed in inverted dishes by using one or two 7-mm threaded extension tubes with a 4× objective (EF 4/0.12), which gave greater working distances. Epifluorescence images were captured in Adobe Photoshop using a Sony DXC-930P 3-chip CCD video camera and F100-MPU integrating frame store. connected to a NuVista+ video digitizer in an Apple Macintosh computer.

B. Confocal Imaging

GFP-expressing *Arabidopsis* seedlings were removed from the agar media and simply mounted in water under glass coverslips for microscopy. Growing roots could also be directly viewed through coverslip-based vessels. The specimens were examined using a BioRad MRC-600 laser-scanning confocal microscope equipped with a 25-mW krypton–argon or argon-ion laser and filter sets suitable for the detection of fluorescein and Texas Red dyes (BioRad filter blocks K1/K2 with the krypton–argon-ion laser. and A1/A2 with the argon-ion laser). We routinely use a Nikon 60× PlanApo NA 1.2 water-immersion objective to minimize loss of signal through spherical aberration at long working distances. For the collection of time-lapse images. the laser light source was attenuated by 99% using a neutral density filter, the confocal aperture was stopped down. and single scans were collected at 2-s intervals. The large data files were transferred to an Apple Macintosh computer, and the programs PicMerge. authored by Eric Sheldon, and 4DTurnaround, authored by Charles Thomas. were used with Adobe Photoshop and Premiere to produce QuickTime movies for display and analysis. More examples of captured images and movies are available on a Web site, which can be accessed at http://www.mrc-lmb.cam.ac.uk.

Direct visualization of GFP fluorescence in living tissues is not prone to fixation or staining artifacts. and can provide images of exceptional clarity. Moreover, the activities of living cells. such as cytoplasmic streaming, are clearly evident during microscopy. Ordinarily, movement within a sample is a nuisance, placing constraints on the use of sometimes lengthy techniques for noise reduction during confocal microscopy. such as frame averaging. However, it is also possible to monitor dynamic events by time-lapse confocal microscopy, and this combination of a vital fluorescent reporter with high-resolution optical techniques shows much promise for use in cell biological and physiological experiments. We have also found that autofluorescent chloroplasts, normally present in the upper parts of the plant. and certain red fluorescent dyes can provide useful counterfluors for GFP. For example, propidium iodide can be applied to live seedlings in water. to specifically label root cell walls and allow accurate identification of GFP expressing cells.

Protocol: Fluorescent Counterstaining

A. Labeling Root Meristem Cell Walls with Propidium Iodide

Arabidopsis seedlings were grown in sterile culture, removed from agar media. and placed in a well of a microtiter dish with 1 ml of staining solution for 10–

20 min at room temperature. An aqueous 10 μg/ml solution of propidium iodide (Sigma) was used to stain the cell walls of the *Arabidopsis* root meristem. The seedlings were then mounted in water under a coverslip for direct microscopic observation. Propidium iodide is red fluorescent and can be detected using a filter set suitable for Texas Red fluorescence. with little spillover between the GFP (fluorescein) and propidium iodide channels. The cationic dye does not readily cross intact membranes, yet it penetrates throughout the meristem and binds to cell walls, forming an outline of the living cells. The dye is excluded by the Casparian strip present in older parts of the root and does not penetrate shoot tissue well. and thus it is best suited for use in the root meristem. An example is shown in Fig. 2A.

B. Labeling Plasma Membranes with FM 1-43

The cationic styrylpyridinium dye FM 1-143 (Molecular Probes Inc.) provides a useful stain for the plasma membrane in root and shoot tissue of *Arabidopsis*. It is particularly useful for specifically labeling the plasma membrane of shoot epidermal cells, and we have been using this to characterize GFP expression patterns in *Arabidopsis* cotyledons and leaves. The seedlings were removed from sterile culture and placed in 1 ml of 1 μg/ml FM 1-43 in water for 10 min at room temperature. They were mounted in water under a coverslip for direct microscopic observation. FM 1-43 emits a broad orange fluorescence. and the signal can be detected in both the red and green emission channels. We generally use 488-nm laser light to excite both GFP and FM 1-43. and to collect the emissions of both fluors in the same channel. This is possible because of the very localized distribution of Fm 1-43 in shoot epidermal cells. An example is shown in Fig. 2B (see color plate).

IV. Marking Different Cell Types in *Arabidopsis*

It is now possible to genetically mark cells within a living organism using GFP and to visualize these cells directly during development. To provide such markers for work in *Arabidopsis* and to allow genetic manipulation of cells during meristem development. we have adapted a scheme for targeted gene expression used in *Drosophila* (Brand and Perrimon. 1993). Brand and Perrimon used a P-element-based "enhancer-trap" strategy to generate *Drosophila* lines that express different patterns of the yeast transcription activator. GAL4. A chosen target gene could then be placed under the control of GAL4 upstream activation sequences (UASs). transformed, and maintained silently in the absence of GAL4. Genetic crosses between this single line and any of the library of GAL4-containing lines could specifically activate the target gene in a particular tissue or cell type. The phenotypic consequences of misexpression. including those lethal to the organism, could then 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. Elevated A/U

content plays a major role in intron recognition during plant pre-mRNA splicing, and we have found that this can poison expression of heterologous A/T-rich genes such as *GAL4* and *GFP*. It was necessary to alter the codon usage of the gene and to use a derivative, *GAL4-VP16*, to ensure efficient expression in *Arabidopsis*. We have randomly inserted the modified *GAL4-VP16* gene into the *Arabidopsis* genome, using *Agrobacterium tumefaciens*–mediated transformation. Expression of the *GAL4-VP16* gene is dependent upon the presence of adjacent genomic enhancer sequences, so different patterns of expression are generated. The inserted DNA also contains a GAL4-responsive *mGFP5* gene (Fig. 3A, see color plate), so patterns of *GAL4-VP16* gene expression are immediately detectable, with each GAL4-expressing cell marked by green fluorescence.

In vivo detection of GFP can be used as a simple genetic screening procedure for plants growing in normal culture, and we have used the marker to greatly speed and improve a large enhancer-trap screen. Because our particular interest lies in the cells of the *Arabidopsis* root tip, we have modified the plant transformation protocol to include an auxin induction of roots from regenerating shootlets. More than 7500 transformants were then generated, planted in grid patterns in sterile culture dishes, and directly screened for GAL4-mediated GFP expression within roots. Several hundred lines with interesting patterns of root expression were choosen, documented, transferred to soil, and grown to seed, to both amplify and self-hybridize the lines. As a result, we have a collection of 250 *Arabidopsis* lines with distinct and stable patterns of GAL4-VP16 and mGFP5 expression in the root (Fig. 3B, see color plate). These are being made available through the *Arabidopsis* stock center, and we have created a graphical database of the expression patterns to allow easy computer and Web access (http://www.mrc-lmb.cam.ac.uk). These lines provide a valuable set of markers in which particular cell types are tagged and can be visualized with unprecedented ease and clarity in living plants. The physical arrangements of cells and common fields of gene expression are highlighted within the root tip. More important, GAL4-VP16 expression within these same lines will allow precise targeted gene misexpression. A chosen target gene can be cloned under the control of GAL4 UASs, transformed, and maintained silently in the absence of GAL4. Genetic crossing of this single line with any of the library of GAL4-containing lines allows specific activation of the target gene in particular tissue and cell types. The phenotypic consequences of misexpression, including those lethal to the organism, can be conveniently studied. We have targeted the expression of toxic and regulatory proteins to particular cells of the root meristem.

V. Spectrally Distinct Fluorescent Proteins for Multichannel Confocal Microscopy

Subcellular structures can be decorated *in vivo* with GFP-tagged proteins and observed microscopically. These benefits would be greatly extended if we could

use additional and distinct fluorescent protein markers in our experiments. This would immediately enable one to examine in detail the dynamic behavior of a GFP-tagged protein or cell with respect to another fluorescent structure.

Several spectral variants of GFP have already been described (Chapter 2 of this book: Heim *et al.*, 1994; Ormo *et al.*, 1996). We have been anxious to obtain one or more new fluorescent protein markers that can be used with GFP and that can be used for dual-channel confocal imaging. A major constraint in choosing a second fluorescent marker is that its excitation should be compatible with commonly used (i.e., cheap) laser sources. Thus, we have experimented with variants of GFP that have yellow-shifted emission (YFP, excitation maximum 514 nm, emission maximum 527 nm) and cyan-shifted emission (CFP, excitation maximum 440 nm, emission maximum 485 nm).

To produce GFP variants that are expressed well in plants, we have modified the genes so that they also contain altered codon usage and mutations that confer improved folding properties, and this has resulted in the production of mYFP (S65G, S72A, V163A, I167T, S175G, T203Y) and mCFP (Y66W, V163A, S175G). The mCFP and mYFP variants are comparable in brightness to our best GFPs but possess quite distinct spectral properties (Fig. 4, see color plate). Although the emission spectra of mCFP, mGFP5, and mYFP overlap to a large degree, laser scanning confocal microscopy allows the use of monochromatic light for selective excitation of the proteins. For example, common multiline argon-ion lasers emit light mainly at discrete wavelengths of 458, 477, 488, and 514 nm, and we have adapted suitable laser line excitation and emission filters for the detection of the GFP variants. An example is provided later in which we have used this technique to visualize the subcellular localization of particular homeodomain proteins in transgenic *Arabidopsis* plants.

The GAL4 system can be used to view the dynamics of protein localization in living plants. We have cloned the cDNA for a homeodomain protein, KNAT3 (Serikawa *et al.*, 1996), from the C24 ecotype of *Arabidopsis* (this is the ecotype that we use for GAL4-directed misexpression), and have generated transgenic plants containing the gene under the control of GAL4-responsive or constitutive promoters. We have fused mYFP to the KNAT3 protein. GAL4-directed expression of the fusion protein in intact plants has shown that subcellular localization of fusion proteins is precisely regulated. The proteins initially accumulate within the cytoplasm of meristematic cells but are progressively concentrated within nuclei as the cells age (Fig. 5, see color plate). KNAT3 is a putative transcription factor that would not be active if it were excluded from the nucleus, and there appears to be clear posttranslational regulation of the protein's activity. Interestingly, transcription of the KNAT3 promoter seems limited to those cells in which the mYFP-protein fusion is nuclear localized (Serikawa *et al.*, 1997). We have now mutagenized the KNAT3 sequence and are screening for mutant forms that escape this regulation, and that should provide dominant gain of function phenotypes.
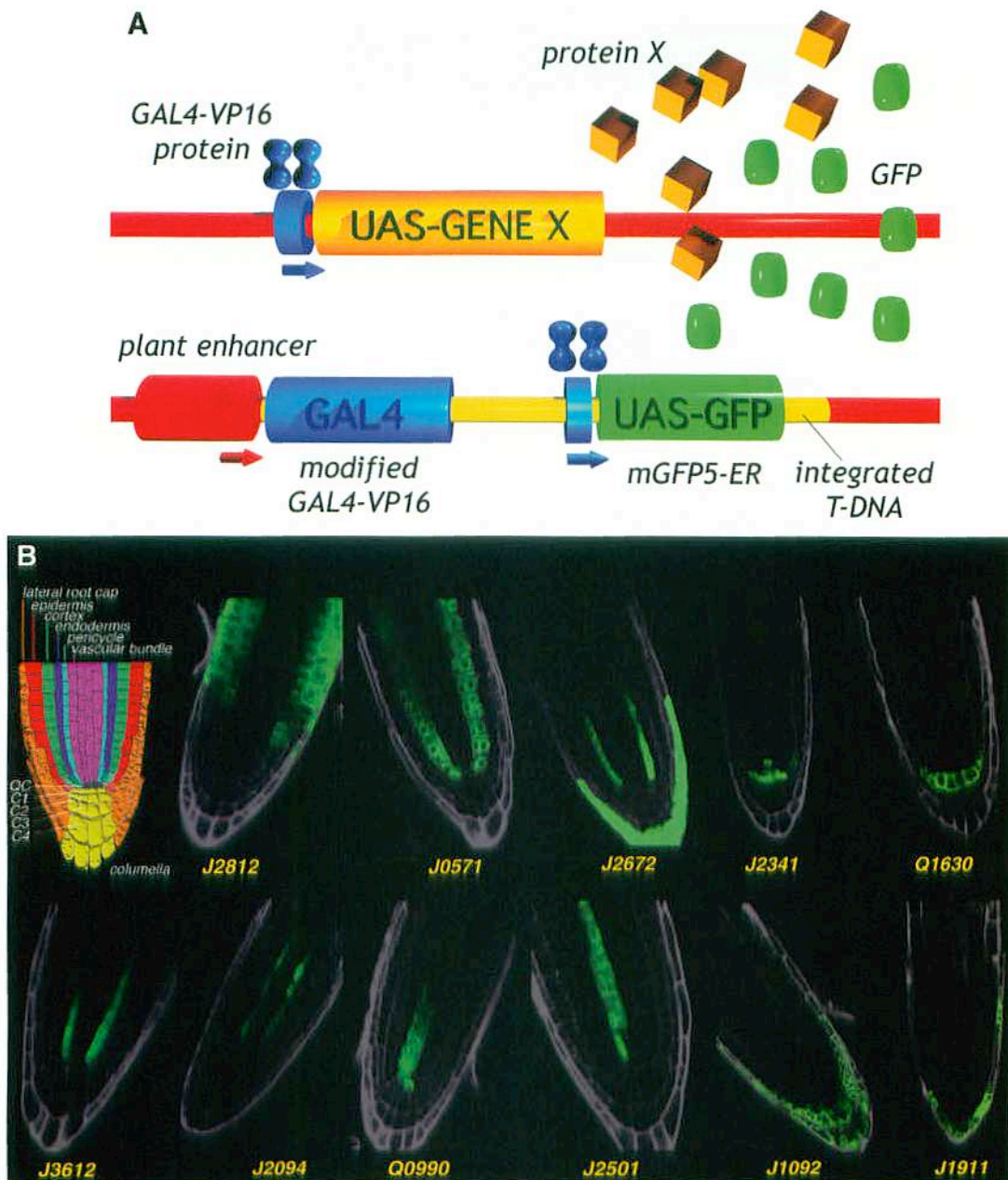
Ch.9, Fig. 3 *Arabidopsis* GAL4-VP16 enhancer trap lines. (A) *Agrobacterium*-mediated plant transformation was used to randomly integrate an engineered T-DNA vector into *Arabidopsis*. The vector contains a *GAL4-VP16* gene with modified codon usage adjacent to a naive promoter at a border of the transferred DNA, a kanamycin resistance selection marker and a GAL4-responsive *mGFP5-ER* gene. The modified GFP has improved fluorescent properties and is targeted to the endoplasmic reticulum. Cell-specific activation of the *GAL4-VP16* gene by a cellular enhancer results in the expression of the GFP marker gene, allowing the simple characterization of expression patterns. Targeted expression of another gene (X) can be induced by a genetic cross with a *GAL4-VP16* line. (B) Confocal micrographs of selected enhancer trap lines showing root-tip-specific expression. The roots were counterstained with propidium iodide, which outlines all live cells. The *GAL4-VP16*-driven GFP signal is shown superimposed. A schematic diagram of the different types of cell in the root tip is shown (upper left).
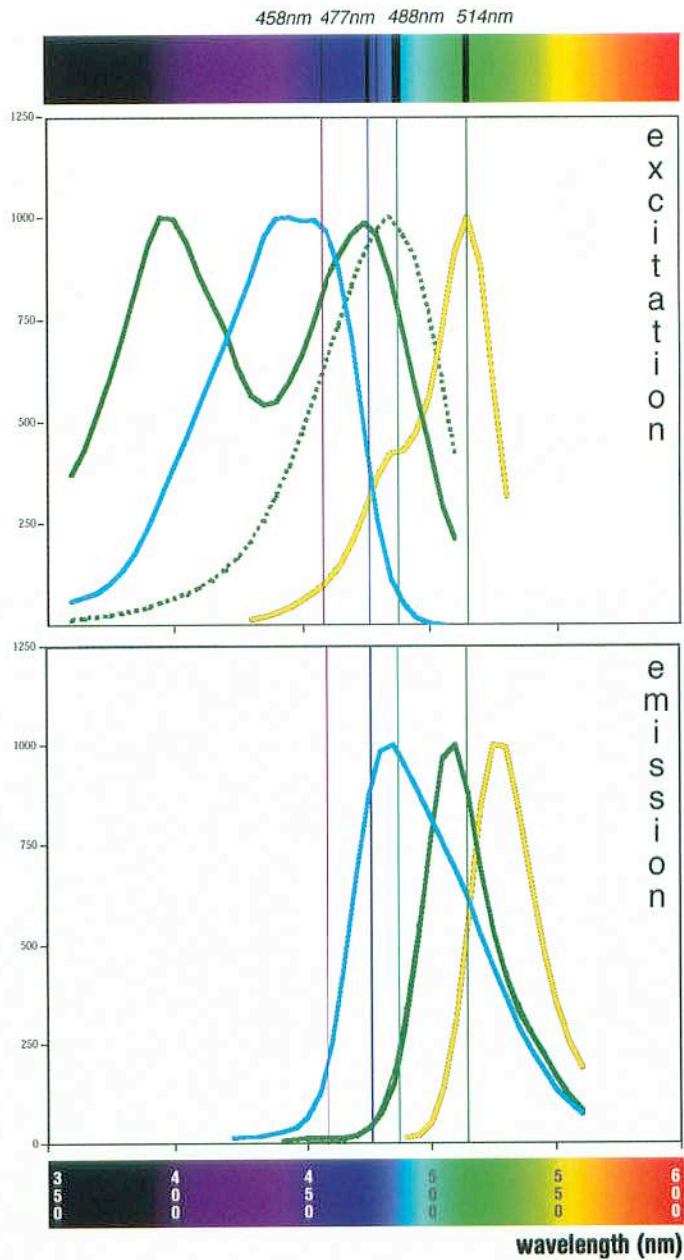
Ch.9, Fig. 4 Fluorescence spectra for variant GFPs. The excitation and emission spectra of cyan, green, and yellow fluorescent protein variants are indicated in their respective colors. The spectra of mGFP5 shown in solid green lines, and those of a green fluorescent variant containing the widely used S65T mutation (mGFP6; J.Hasselhoff, unpublished results) are shown as green dashed lines. The main laser lines for an argon-ion laser are indicated by vertical tracing.
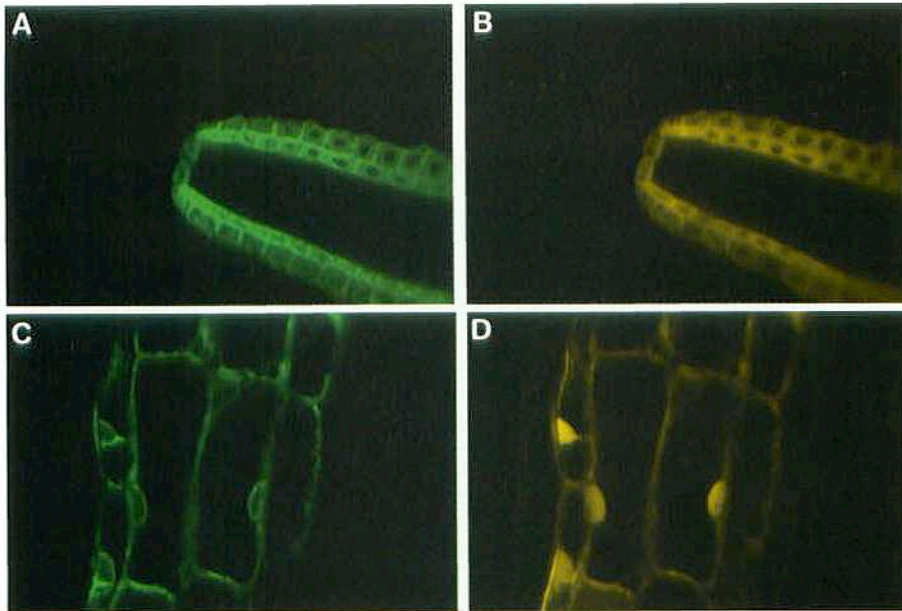
Ch.9, Fig. 5 Dual-channel confocal imaging of GFP variants. The GAL4-VP16 enhancer trap line J0571 was used to drive expression of a KNAT3–yellow fluorescent protein fusion in the endodermis and cortex of the *Arabidopsis* root. The KNAT3 fusion protein is localized in the cytoplasm of meristematic cells (B), but accumulates within the nuclei of older cells (D). The perinuclear and endomembrane distribution of ER-localized green fluorescent protein is also shown (A, C).
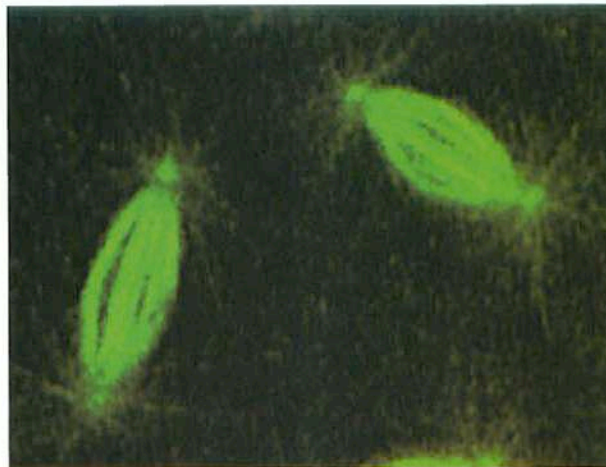
Ch.10, Fig. 2 Mitotic spindles labeled with Ncd-GFP. Ncd-GFP forms pole-to-pole fibers along the spindle microtubules, which are especially prominent in metaphase and early anaphase. The distribution of Ncd-GFP differs from that of tubulin during metaphase and early anaphase in that the fibers of Ncd-GFP extend across the chromosomes rather than terminating at the chromosomes positioned on or just moving off the metaphase plate. The spindles are in early anaphase of mitotic cycle 9. The centrosomes and astral microtubules are also labeled with Ncd-GFP.
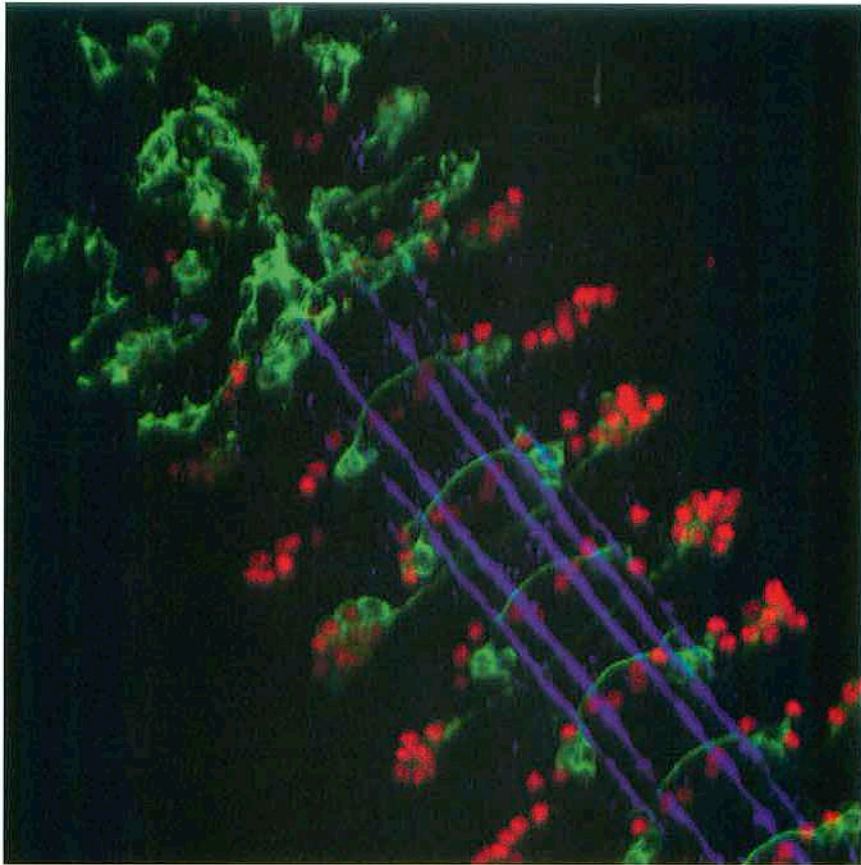
Chapter 11, Figure 5 Targeted expression of tau–GFP (green) labels individual neurons as they extend their axons in the central nervous system of living embryos. Tau–GFP continues to fluoresce after fixation, which allows immunolabeling of other proteins, such as Even-skipped (red) and Fasciclin II (blue).

Protocol: Double Labeling with GFP Variants

    1. Plants expressing mCFP, mGFP5, and/or mYFP proteins were grown in sterile culture and mounted in water for microscopy.

    2. A Biorad MRC-600 microscope was equipped with an 80-mW argon ion laser and a motorized excitation filter wheel containing narrow bandpass filters to select laser lines at 458 nm for excitation of mCFP, 477 nm for excitation of mGFP5 (mGFP5), and 514 nm for excitation of mYFP. A multiline argon-ion laser of higher power (>50 mW) is generally needed to provide 458-nm illumination of useful intensity. We have used mCFP and mYFP, and mGFP5 and mYFP together for double-labeling experiments. The proteins were sequentially excited using the appropriate laser lines, and the signals were collected through specialized emission filter blocks: mCFP/mYFP = 495-nm longpass dichroic mirror, 485 ± 30 nm, 540- ± 30-nm bandpass filters, or mGFP5/mYFP = 527 nm longpass dichroic mirror, 500-nm longpass and 540- ± 30-nm bandpass filters (Omega Optical). The use of selective monochromatic excitation allows useful discrimination between mGFP5 and mYFP, which have overlapping fluorescent spectra. The greater spectral differences between mCFP and mYFP result in clean discrimination of the fluorescent signals. Sequentially collected images were merged and pseudocolored using Adobe Photoshop.

VI. Summary

    Unlike enzyme markers, green fluorescent protein can be visualized at high resolution in living cells using confocal microscopy. The images are not prone to fixation or staining artifacts, and can be of exceptional clarity. Moreover, the activities of living cells, such as cytoplasmic streaming, are clearly evident during microscopy. Ordinarily, movement within a sample is a nuisance, placing constraints on the use of sometimes lengthy techniques for noise reduction during confocal microscopy, such as frame averaging. However, it is possible to monitor dynamic events by time-lapse confocal microscopy, and this combination of a vital fluorescent reporter with high-resolution optical techniques shows much promise for use in cell biological and physiological experiments.

    Genetic systems such as that of *Arabidopsis* provide a large resource of potentially informative mutants, and there has been much recent improvement in techniques for determining the molecular basis of a particular phenotype. The use of fluorescent proteins will provide further tools for examining the biology of mutant cells. The precision with which particular cellular structures can be decorated with GFP and the ease with which subcellular traffic can be monitored indicate that this approach will be very useful for cell biological and physiological observations, particularly for detailed examination of plant mutant phenotypes.

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