The highly regulated structural components of the plant cell form the basis of its function. It is becoming increasingly recognized that cellular components are ordered into regulatory units ranging from the multienzyme complexes that allow metabolic channeling during primary metabolism to the “transducen” complexes of signal transduction elements that allow for the highly efficient transfer of information within the cell. Against this structural background the highly dynamic processes regulating cell function are played out. Recent technological advances in three areas have driven our understanding of the complexities of the structural and functional dynamics of the plant cell. First, microscope and digital camera technology has seen not only improvements in the resolution of the optics and sensitivity of detectors, but also the development of novel microscopy applications such as confocal and multiphoton microscopy. These technologies are allowing cell biologists to image the dynamics of living cells with unparalleled three-dimensional resolution. The second advance has been in the availability of increasingly powerful and affordable computers. The computer control/analysis required for many of the new microscopy techniques was simply unavailable until recently. Third, there have been dramatic advances in the available probes to use with these new microscopy approaches. Thus the plant cell biologist now has available a vast array of fluorescent probes that will report cell parameters as diverse as the pH of the cytosol, the oxygen level in a tissue, or the dynamics of the cytoskeleton. The combination of these new approaches has led to an increasingly detailed picture of how plant cells regulate their activities.

**Key words:** caged probes; cell biology (plants); confocal microscopy; green fluorescent protein; laser tweezers; light microscopy, ratio imaging; signal transduction.

**OVERVIEW**

The past decade has seen an explosion in the development of new techniques in cell biology, particularly in the field of light microscopy and imaging. New microscopes are continuously being developed that have either improved optics or the ability to image new features of the cell. High performance cameras with improved detection capabilities are also becoming a common feature of many cell biology laboratories. Since its conception by Marvin Minsky in the 1950s, confocal microscopy has now become a routine technique and indispensable tool not only for cell biological studies but also for molecular investigations (Lichtman, 1994). In addition, although used by only a handful of research laboratories worldwide since its recent introduction, applications of multiphoton fluorescence microscopy to biological questions are likely to grow rapidly within the next few years. This technique is poised to provide a very real alternative to confocal microscopy in revealing the three-dimensional dynamics of the plant cell (Denk, Strickler, and Webb, 1990). Computers are also fast evolving to meet the demanding requirements for image capture and analysis that have become essential to make full use of these new instruments. For example, approaches such as real-time computational image deconvolution (Scalettar et al., 1996) were until recently only available to microscopists with access to a supercomputer. All these developments have translated into a repertoire of advanced microscopy and imaging techniques that can be exploited to aid in further understanding the inner workings of plant cells.

The power of modern light microscopy to view plant cellular structure relies almost as much on improving fluorescent probes as it does on faster computers, sensitive photodetectors, and advanced lasers. The green fluorescent protein (GFP), a new cellular marker cloned from the jellyfish *Aequorea victoria*, has allowed investigators to monitor cytoskeletal and organelle dynamics in living plant cells (Kohler, 1998; Kost, Spiegelhofer, and Chua, 1998; Marc et al., 1998) as well as long-distance transport of macromolecules and viruses (e.g., Imlau, Truermit, and Sauer, 1999; Oparka et al., 1999; reviewed by Thompson and Schulz, 1999; Lazarowitz, 1999). Several spectral variants of this protein have also enabled researchers to monitor the dynamics of two or more molecules in the cell simultaneously (Ellenberg, Lippincott-Schwartz, and Presley, 1999; Palm and Wlodawer, 1999; Haseloff, 1999). These spectral variants of GFP have also led to the design of transgenic intracellular ion sensors that take advantage of fluorescence energy resonance transfer (FRET) between different spectral forms of GFP (Miyawaki et al., 1997, 1999; Allen et al., 1999; Gadella, van der Krogt, and Bisseling, 1999).

The combination of sophisticated instrumentation with new fluorescent probes has transformed light microscopy into a powerful analytical tool that has allowed researchers to gain new insights on basic plant cellular structure and function. As new approaches emerge and current technologies undergo refinement, we are constantly presented with a new suite of tools to tackle previously intractable questions in plant cell biology. Due to space limitations, this review will highlight just some of the developments in optical microscopy and imaging technology outlined above and how they have revolutionized our view of plant cell function. We will also suggest some of the...
directions these new tools could lead us as we take plant cell biology into the new millennium.

ADVANCED OPTICAL MICROSCOPY
INSTRUMENTATION AND TECHNIQUES

For more than three centuries, the light microscope has provided biologists with a powerful visual and analytical tool to study cells and tissues. With the introduction of the electron microscope in the 1930s, researchers were able to take cell biology several steps further into understanding cell function by resolving structures not previously visible with the light microscope. Although electron microscopy continues to play an essential role in characterizing cellular function it can only yield static images of cells and tissues. Yet it has always been clear to microscopists that the plant cell possesses an intricately patterned, highly dynamic, three-dimensional structure, which is thought to form the basis of cellular function. Conventional light microscopy in combination with other contrast-enhancing approaches such as differential interference contrast (DIC) microscopy and phase contrast have provided tools to monitor the structural dynamics of the cell (for a full discussion of these techniques, see Ruzin, 1999). These microscopy approaches have continued to be instrumental in cataloging the structural basis of plant cellular processes from Robert Hooke’s discovery in 1665 that cells formed a functional unit of cork to modern high-resolution DIC video-enhanced microscopy that allows individual plant microtubules to be imaged in solution (Moore et al., 1997). However, cell biologists had to await significant technological advances to be able to capture and, more recently, manipulate the dynamic, three-dimensional nature of specific molecular components of the plant cell in vivo.

Confocal microscopy—Confocal microscopy is proving to be one of the most exciting advances in optical microscopy of the last century. Although conventional wide-field epifluorescence microscopy has been a powerful tool for locating specific molecular components of the cell, it suffers from the problem of out-of-focus fluorescence interfering with the contrast and resolution of the final image (Webb, 1999). With the commercialization of confocal microscopes in 1988, biologists began seeing cells in three dimensions and with much more clarity than previously possible. The impact that confocal microscopy has had on cell biology is evident in the number of reviews and books written on the subject (e.g., Pawley, 1995; Sheppard and Shotton, 1997; Webb, 1999). The number of scientific papers employing confocal microscopy in plant biology has also grown dramatically since it was first introduced and is indicative of the importance of this technology to plant science research (Hepler and Gunning, 1998; Wymer et al., 1999).

Confocal microscopes work by exciting fluorescence with a highly focused beam of laser light. The laser illuminates the sample, and the emitted fluorescent light is collected by the microscope objective. Light emitted from the focal plane of the microscope passes through a pinhole aperture positioned in front of a photomultiplier detector. The photomultiplier then passes the detected light signal to a computer, which will use it to generate an image. The critical feature of the confocal approach is that light emitted from points above or below the plane of focus is blocked by the pinhole and so never reaches the detector. Thus only an image of the fluorescence from the focal plane is observed (i.e., “the microscope has generated an ‘optical section’”). A single two-dimensional view of the optical section is then obtained by scanning the laser from point to point over the sample until the entire focal plane of the sample is imaged (Lichtman, 1994). Because only the light at the focal plane of the sample passes through the pinhole onto the detector, the resulting image is free from out-of-focus fluorescence. By collecting a series of optical sections at different focal planes, a full three-dimensional image of the sample is reconstructed (Webb, 1999). As sectioning is performed using optics at different focal planes, a full three-dimensional image of the sample is free from out-of-focus fluorescence. By collecting a series of optical sections taken throughout the depth of the cell the shows the complex pattern of hyphae as they ramify throughout the single colonized cell (Fig. 2). Such an appreciation of the three-dimensional details and complexity that forms the basis of cellular structure is now routinely possible in large part because of widespread availability of confocal microscopes.

Confocal microscopy has been applied to answer questions as varied as determining cellulose microfibril orientation (Verbeelen and Stickens, 1995), in situ localization of RNA and DNA probes (Wymer et al., 1999), determining the spatial and temporal characteristics of cell wall pH during root growth (Taylor, Slatter, and, Leopold, 1996; Bibikova et al., 1998) and for monitoring fluorescent dye movement to study cell-to-cell communication in roots and shoots (Zhu, Lucas, and Rost, 1998; Gisels et al., 1999). Three-dimensional morphological examination of surface structures used to be the realm of scanning electron microscopy (SEM), but confocal microscopy is proving to be an effective tool in these type of studies as well (Lemon and Posluszny, 1998; Melville et al., 1998), with the important advantage of being applicable to living cells. However, it is important to note that the confocal microscope has only reached its place as an indispensable tool for the plant biologist because, in parallel, researchers have been developing an ever increasing range of fluorescent reagents to visualize defined cellular structures and processes (see below).

Multiphoton fluorescence microscopy—A new approach to live cell imaging that is likely to have as dramatic an impact as confocal microscopy is multiphoton microscopy. This technique uses a laser to deliver a burst of low-energy, long-wavelength photons in a short pulse. The photon flux density in the pulses is high enough that some of the photons hit a target fluorochrome simultaneously. When the target absorbs two photons at essentially the same time, they produce a similar effect as a single short-wavelength photon with twice the energy (i.e., half the wavelength). For example, two photons of infrared radiation (e.g., 700 nm) when absorbed simultaneously can excite a UV or blue light excitable dye (e.g., a dye normally excited by 350 nm light; Xu et al., 1995; Sako et al., 1995). The probability that two photons will arrive at the fluorochrome simultaneously drops off dramatically away from the focal plane of the microscope. Thus fluorescence is only effectively excited at the focal plane, and an optical section analogous to a confocal optical section is generated. Unlike the confocal approach, however, the optical section is produced by the excitation of the fluorochrome in a single focal plane. In addition, the multiphoton microscope imposes much less demand on the detector optics because, unlike the confocal microscope, the emitted light does not have to be accurately focused through a pinhole to achieve the sectioning effect. This helps improve the efficiency of detection and the ability to image deep into tissues.

Multiphoton microscopy has the potential to circumvent some of the problems inherent in confocal imaging. The high-intensity, short-wavelength irradiation used to excite the samples in many confocal imaging situations can be damaging when imaged over long periods. Also, the shorter wavelengths of light tend to penetrate less far into a sample (Gilroy, 1997). Multiphoton microscopy, on the other hand, limits excitation to the focal plane of the sample, and, therefore, photobleaching of the fluorochrome outside the focal plane is minimized. This feature, in addition to the fact that the longer wavelength photons used in multiphoton microscopy have less energy, cuts down on phototoxicity to the sample (Denk, Piston, and Webb, 1995). Longer wavelength photons are also less prone to scattering, making it possible to image deeper into the sample (Gilroy, 1997; Fricker and Oparka, 1999). Multiphoton microscopy promises to generate confocal-like images while making longer observation times possible and should provide an important complement to the confocal microscope (Sako et al., 1995).
FLUORESCENT DYES

The new imaging technologies have provided unparalleled opportunities to image the plant cell. However, the insights they have revealed have resulted in large part because of the explosion in the availability of optical probes that can be used with these techniques. Chemists are continuously developing new fluorescent labels to allow imaging of proteins, nucleic acids, lipids, and just about any specific cell component. In addition, targeted probes for organelles and even specific ions are now commercially available (Haugland, 1999). Many of these reagents have yet to be successfully applied to plant cells, and we can expect some plant-specific problems with the use of some probes. For example, although fluorescent probes to image membrane potential are widely used in animal cells (e.g., Montana, Farkas, and Loew, 1989; Haugland, 1999), plant biologists have had little success with equivalent approaches. Similarly, the ubiquitous “ester-loading” method employed to load fluorescent dyes into the animal cell cytoplasm has had very limited success in plants where dyes may completely fail to penetrate the cell (Gilroy, 1997; Fricker et al., 1999) or accumulate in the vacuole rather than the cytoplasm (Swanson and Jones, 1996). However, there is a vast array of untried fluorescent probes available to the plant biologist, and their potential to reveal specific insights into how the plant cell functions is enormous. This is especially true when they are combined with image analysis approaches, such as ratio imaging, which allows fluorescence images to yield quantitative information about the dynamics of the cell.

Fluorescence ratio imaging. —The fluorescence of many fluorochromes is sensitive to their molecular environment. For example, the fluorescence of perhaps the most widely used fluorochrome, fluorescein, increases as the pH rises. This has led to the design of a whole spectrum of dyes that are selective indicators of specific cell parameters or molecules. Thus there are dyes whose fluorescence reports parameters ranging from lipid mobility to specific ion concentrations (e.g., Ca$^{2+}$, Mg$^{2+}$, H$^+$, K$^+$, Na$^+$, Cl$^-$, Zn$^{2+}$; Haugland, 1999). These indicators have driven much of the advances in our understanding of cellular control as they provide an image of the dynamics of a well-defined cell component. Their usage is perhaps most prevalent in the study of ionic signals such as Ca$^{2+}$ and pH. For example, Ca$^{2+}$-selective dyes such as Fluo-3 and Calcium Green, whose fluorescence increases in response to Ca$^{2+}$ levels, have been instrumental in demonstrating a previously unsuspected role for Ca$^{2+}$ release from intracellular stores and intracellular waves of Ca$^{2+}$ in pollen tube growth (reviewed in Franklin-Tong, 1999). However, there have always been limitations in interpreting fluorescence images from such dyes. The signal from the dye may increase due to increased Ca$^{2+}$, but also due to dye relocalization, differences in dye concentration, photobleaching, and differences in optics between regions of a tissue or even subregions of a cell. This limitation was elegantly overcome by applying the ratio analysis approach (Tanasagurn et al., 1984; Tsien and Poenie, 1986). In this approach a spectral shift in fluorescence is monitored rather than simply monitoring fluorescence intensity. A wavelength of fluorescence that increases with ion concentration is compared with one that is invariant or decreases with ion level. The ratio of these intensities is independent of dye concentration and many of the other optical artifacts that make the signal from single-wavelength dyes hard to quantify accurately.

The ratio analysis approach has allowed reliable, quantitative, spatial, and temporal analysis of images from fluorescent probes. Pollen tube growth and signaling in guard cells serve to highlight the power of this technology and the biological surprises it has revealed. Ratio imaging has shown a tip-focused gradient in Ca$^{2+}$ centered on the growing tip of the pollen tube (Fig. 3). The gradient is always associated with the growing tip and altering the gradient redirects growth (Malho and Trewavas, 1996; Bibikova, Zhigilei, and Gilroy, 1997). When tip growth ceases, the gradient is lost. Such data led to the idea that localized Ca$^{2+}$ influx across the plasma membrane generated a tip-focused Ca$^{2+}$ gradient that promoted secretion...
Figs. 3–6. Optical probes used in modern light microscopy to visualize cellular dynamics. 3. Ratio imaging of cytoplasmic Ca\(^{2+}\) levels in a pollen tube of *Trandescantia*. The pollen tube was microinjected with Indo-1 and Ca\(^{2+}\) levels determined from the emission ratio 400–430 nm/460–480 nm using a Zeiss LSM 410 UV confocal microscope. Ca\(^{2+}\) levels were pseudocolored according to the inset scale. Fluorescence ratio imaging reveals the highly localized calcium gradient at the pollen tube tip. Scale bar = 10 \(\mu\)m. 4. Transient expression of GFP in an epidermal cell of *Nicotiana tabacum*. The coding region of enhanced GFP (eGFP) was linked to the cauliflower mosaic virus 35S promoter, biolistically bombarded, and the leaf imaged using a Bio-Rad 1024 confocal microscope. Free GFP is distributed throughout the cell cytoplasm and can also be seen to enter the nucleus (n) but not the chloroplasts (c) (S. Yin, E. B. Blanchaflor, and N. L. Paiva, unpublished data). Scale bar = 20 \(\mu\)m. 5. A leaf of *Nicotiana benthamiana* inoculated with tobacco mosaic virus that was genetically modified to express GFP. The leaf shows green fluorescence associated with the veins when photographed under UV light. The advent of GFP technology has allowed the visualization of the pathways by which viruses move in living plants (N. Cheng and R. S. Nelson, unpublished data). Bar = 1 cm. 6. Reorientation of *Trandescantia* pollen tube growth after local photoactivation of caged Ca\(^{2+}\) ionophore (A23187). Uncaging of the ionophore was accomplished by UV irradiating the region denoted by the white box using a 0.7-s scan of a Zeiss UV confocal microscope. Local activation of the ionophore allows Ca\(^{2+}\) influx into the cell at that point. The pollen tube reorients toward the new site of elevated calcium induced by the localized Ca\(^{2+}\) influx. Scale bar = 10 \(\mu\)m. Inset shows fluorescence ratio imaging of Ca\(^{2+}\) levels in *Trandescantia* pollen tubes during localized photoactivation of caged Ca\(^{2+}\) ionophore (Bibikova et al., 1997). The pollen tube was microinjected with calcium green/bodamine and Ca\(^{2+}\) levels pseudocolored according to the scale in Fig. 3. Note that Ca\(^{2+}\) is elevated at the site of ionophore uncaging. Scale bar = 10 \(\mu\)m.
and therefore drives growth. The link between the \( \text{Ca}^{2+} \) gradient and growth was even more strongly made when it was noted by several researchers that pollen tubes grow in pulses and that the gradient also oscillated (reviewed in Franklin-Tong, 1999). However, a closer examination revealed that the story is not so simple. The increase in the \( \text{Ca}^{2+} \) gradient coincides with growth but comparing this to measurements made using the self-referencing (vibrating) microelectrode showed \( \text{Ca}^{2+} \) influx into the tip lagged the elevation of the cytosolic gradient by 10 s (Pierson et al., 1996; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). We are still trying to understand how these observations fit into a model of the regulation of tip growth, but \( \text{Ca}^{2+} \) incorporation into newly synthesized wall polymers, stretch-activated \( \text{Ca}^{2+} \) channels, and \( \text{Ca}^{2+} \) release from internal stores all seem plausible features that could be interacting to give these ion dynamics. Recent imaging data suggest the \( \text{Ca}^{2+} \) gradient is controlled in part by the \( \text{Ca}^{2+} \) ATPase (reviewed in Li et al., 1999) and possibly influenced by the microtubule cytoskeleton (Bibikova, Blancaflor, and Gilroy, 1999). Thus the ratio imaging data have not only indicated that \( \text{Ca}^{2+} \) is a critical player in the growth machinery but has also shown us that we have only just begun to characterize its action.

In guard cells, the subtlety of potential signaling systems is dramatically revealed by the ratio analysis approach. Guard cells respond to multiple environmental signals including red and blue light, \( \text{CO}_2 \), humidity, and the stress hormone ABA (abscisic acid; Assmann and Shimazaki, 1999). This ability to integrate numerous signals has led to intensive studies on signaling in these cells. Studies with inhibitors of \( \text{Ca}^{2+} \) signaling hinted at a \( \text{Ca}^{2+} \)-dependent system of signaling. However, only after ratio imaging allowed the spatial and temporal dynamics of the system to be seen and manipulated was the full extent of the complex signaling network in this single cell revealed (reviewed in Hetherington et al., 1998). These studies showed the operation of \( \text{Ca}^{2+} \)-dependent and \( \text{Ca}^{2+} \)-independent signaling systems in response to ABA that is influenced by the environmental history of the plant (Allan et al., 1994). Evidence has also accumulated for information being encoded in the spatial patterns, amplitude, and frequency of transient elevations in \( \text{Ca}^{2+} \) levels occurring within the guard cell cytoplasm (Gilroy, Read, and Trewavas, 1990; Grabov and Blatt, 1998; Staxen et al., 1999). For example, Staxen et al. (1999) presented evidence that the frequency of \( \text{Ca}^{2+} \) transients might encode the ambient ABA concentration. Recent work with calmodulin-dependent protein kinase II from mammalian cells shows a single enzyme can decode \( \text{Ca}^{2+} \) transients into specific frequency-related intermediate enzymatic activities (Dekoninck and Schulman, 1998), providing a model of how plant cells might use such frequency-related information. It is clear that most cells respond to myriad signals with a remarkable ability to integrate and respond appropriately. We can anticipate that the guard cell story of a complex and dynamic regulatory network will be reprised in many other systems as more plant cells receive equivalent, intensive analysis by cell physiologists.

**Aequorin**—No review of imaging and analysis of signaling systems in plants would be complete without mentioning the advances made in the use of aequorin as a luminescent \( \text{Ca}^{2+} \) indicator. Aequorin, like GFP, is a protein originally from the jellyfish *A. victoria* that has proven of immense use to cell biologists. Aequorin is a luminescent protein consisting of an apoprotein (apoaequorin) and coelentrazine, its small cofactor. Light emission from aequorin is triggered by \( \text{Ca}^{2+} \) with the rate of luminescence being proportional to the \( \text{Ca}^{2+} \) concentration. The gene for aequorin has been cloned, and when cells expressing this protein are treated with coelentrazine, functional aequorin is reconstituted. The luminescence rate can be calibrated to an absolute \( \text{Ca}^{2+} \) concentration making it possible to use transgenically expressed aequorin as a \( \text{Ca}^{2+} \) sensor. Plants such as tobacco and *Arabidopsis* have been stably transformed with aequorin and their \( \text{Ca}^{2+} \) dynamics monitored either by putting whole plants in a luminometer, or by visualizing light emission using a highly sensitive camera.

Aequorin has revealed some surprising insights into plant signaling and regulation. It has been used to show that there are proliferating waves of \( \text{Ca}^{2+} \) and organ-specific responses when challenged by a host of environmental signals such as salinity, drought, cold, heat shock, blue light, elicitors, touch, or anoxia (reviewed in Trewavas, 1999). With aequorin it was also shown that there are oscillations in \( \text{Ca}^{2+} \) associated with the circadian rhythms of the plant (Johnson et al., 1995). Aequorin has been targeted to various organelles and has shown, for example, that not only is chloroplast \( \text{Ca}^{2+} \) regulated independently of the cytosol (Johnson et al., 1995), but also, that despite the extensive pore system in the nuclear membrane, nuclear \( \text{Ca}^{2+} \) is also regulated independently of cytosolic levels (Van der Luit et al., 1999).

Artificial coelentrazines of varying \( \text{Ca}^{2+} \) affinities, aequorins with various spectral properties, and even a ratioable aequorin are available (Knight et al., 1993). However, imaging the rapid dynamics of \( \text{Ca}^{2+} \) at the cellular level using aequorin is very difficult, and imaging with subcellular resolution has proven impossible (unless the protein is specifically targeted to a subcellular site) due to the inherently low signal generated by this luminescent protein. These applications are more suited to analysis by fluorescent dyes and GFP-based sensors such as the cameleon \( \text{Ca}^{2+} \) probe (see below). However, aequorin has a far superior dynamic range than these fluorescent sensors and can therefore easily detect rapid \( \text{Ca}^{2+} \) transients in whole plants. Aequorin’s high signal-to-background also makes it usable in plant cells where autofluorescence makes fluorescent sensors inapplicable. These advantages and disadvantages of aequorin highlight that although there is a remarkable array of probes and technologies available to the plant biologist to monitor cell behavior, each is appropriate to answer a specific kind of question and it is in their combination and complementary usage that the real power of these new approaches lies.

**GREEN FLUORESCENT PROTEINS: NOVEL INSIGHTS ON CELLULAR STRUCTURE, DYNAMICS, AND SIGNALING IN LIVING PLANT CELLS**

The discovery of GFP and several of its spectral variants from *Aequorea victoria* (Ellenberg, Lippincott-Schwartz, and Presley, 1999; Palm and Wlodawer, 1999; Haseloff, 1999) and more recently a red fluorescent protein from another anthozoan species (Matz et al., 1999) has provided a unique complement to the battery of imaging devices that have been developed during the latter part of the 20th century. GFP is a 27-kDa protein that fluoresces bright green when excited by UV or blue light (Fig. 4). GFP fluorescence depends on an internal chromophore and so, unlike other biological fluorochromes, it does not need the addition of a cofactor to fluoresce (Cubbit,
The plant cytoskeleton—Through the use of highly specific antibodies against cytoskeletal proteins, researchers were able to infer from fixed, static images how the plant cytoskeleton reorganizes during various developmental stages and in response to a variety of environmental stimuli (Lloyd, 1987; Cyr, 1994; Hush and Overall, 1996; Nick, 1998). A more dynamic element was added to this snapshot view of the cell through the use of fluorescent analog cytochemistry, whereby a fluorescently labeled protein (e.g., rhodamine-tubulin) is introduced into the cell by microinjection. The labeled protein could then be incorporated into the pool of normal cellular proteins and their behavior monitored by fluorescence or confocal microscopy. Using this technique, the highly dynamic nature of both the cortical and spindle microtubule cytoskeleton of living plant cells were directly observed and quantified (Wasteney, Gunning, and Hepler, 1993; Hush et al., 1994; Yuan et al., 1994; Wymer et al., 1997; Himmelspach et al., 1999). Similar progress has been made with the actin cytoskeleton. By microinjecting small amounts of fluorescently labeled phalloidin, a low molecular weight fungal metabolite that binds preferentially to F-actin, it was possible to observe the actin cytoskeleton in living plant cells undergoing division (Schmit and Lambert, 1990; Cleary, 1995), in pollen tubes (Miller, Lancelle, and Hepler, 1996), and in *Transdescantia* stamen hairs (Ren et al., 1997).

The procedures to load fluorescently labeled proteins into cells, however, can be a technically demanding and slow process, especially for walled plant cells. Microinjection, being an invasive technique, can also perturb cellular function. Furthermore, the technique suffers from limited observation times and is only applicable to isolated or exposed single cells (Kost, Mathur, and Chua, 1999). These limitations have been partially overcome with the use of GFP. By fusing the microtubule-binding domain of the microtubule-associated protein 4 (MAP4) with GFP and transiently expressing the recombinant protein in plant cells, Marc et al. (1998) were able to label cortical microtubules in living epidermal cells. A course analysis of the labeled microtubules revealed intricate dynamics including localized reorientations, lengthening, shortening, and translocation of microtubules.

Kost, Spielhofer, and Chua (1998) have taken a similar approach for actin by transiently expressing GFP fused to the actin binding domain of talin, a mammalian actin-binding protein (designated GFP-mTn). GFP-mTn constructs decorated actin filaments in tobacco pollen tubes and cultured BY2 cells, which allowed the in vivo observation of actin dynamics in these cells. More recently, stable transformation of Arabidopsis with the GFP-mTn and GFP-MAP4 fusions was used to monitor actin filament and microtubule dynamics during trichome development (Mathur et al., 1999; Mathur and Chua, 2000).

Although these are the only reports to date on visualizing the cytoskeleton in living plant cells using GFP fusions, we can anticipate that studies on the plant cytoskeleton will advance rapidly in the next few years because of this technology. Additional advances will come by applying multiphoton microscopy to intact plants expressing the GFP-cytoskeleton binding protein fusions to image cytoskeletal dynamics in cells located deeper in an intact plant organ (Potter et al., 1996; Schwille et al., 1999). Studies with fluorescent analog cytochemistry already suggest that microtubules on different faces of a cell may have vastly different dynamics and organization (Yuan et al., 1992), and studies on fixed material have shown that the cytoskeleton in the innermost cells of roots reorganizes rapidly when exposed to a variety of hormones and other stimuli (Shibaoka, 1994; Blancaflor and Hasenstein, 1995a, b; Blancaflor, Jones, and Gilroy, 1998; Nick, 1998; Hasenstein, Blancaflor, and Lee, 1999). Imaging the cytoskeleton deep into living tissues using multiphoton and GFP technology should reveal the dynamic nature of these responses. Furthermore, the availability of several spectral variants of GFP will be of enormous value in allowing multiple tagging experiments to probe the interaction between different components of the plant cytoskeleton in living cells. For example, studies of the dynamics of the interaction between microtubules and actin should be high on the priority list of plant cytoskeletal researchers (Tomina et al., 1997; Collings et al., 1998).

Visualizing intracellular organelle dynamics and endomembrane trafficking—The use of GFP has also led to new insights on the dynamics and functions of various cellular organelles and the endomembrane system of plants. The creation of GFP fusions that contain specific localization sequences results in the retention of GFP in specific organelles and membrane systems and allows their direct observation in vivo (Kohler, 1998). Although membrane-permeable fluorescent dyes have been available to observe the dynamics of some cellular components such as the endoplasmic reticulum (ER) and mitochondria in living plant cells, questions as to their specificity have been a major concern (Hepler and Gunning, 1998). Furthermore, rapid photobleaching and the possible toxic effects of these fluorescent dyes have made long-term observation of these organelles in living plant cells difficult (Scott et al., 1999).

So far, the most common and readily available GFP-based subcellular markers are constructs that have the ER peptide retention signal, KDEL. By fusing the KDEL sequence to the coding sequence of GFP and expressing this construct in plants, the dynamic cortical network of membranous tubules representing the ER could be visualized (Boevink et al., 1996, 1998). This new vital ER marker has already provided new information on structure/function of the endomembrane system as well as vesicle trafficking in plants (Hawes, Brandizzi, and Andreeva, 1999). For example, GFP fused to the ER re-
tention signal has not only demonstrated the highly dynamic nature of the plant endomembrane system, but has also revealed the existence of rapidly moving organelles (Haseloff and Siemering, 1998). Although these organelles have been suggested to be proplastids, an alternative explanation as to the identity of these organelles has been proposed (Gunning, 1998). Using a GFP fusion targeted to the Golgi apparatus (made by linking GFP to the transmembrane domain of a rat sialyl transferase) in combination with a second construct consisting of GFP fused to the Arabidopsis H/KDEL homologue of the yeast HDEL receptor, aERD2, the simultaneous visualization of the Golgi and ER was possible. This provided the first evidence that the ER and Golgi in plants are closely associated (Boevink et al., 1998).

In addition to the ER and Golgi, GFP has also been targeted to the mitochondria (Kohler et al., 1997b), vacuole (Sansebastiano et al., 1998), plastids (Kohler et al., 1997a; Tirlapur et al., 1999), and the nucleus (Chytilova, Macas, and Galbraith, 1999). Among other findings, this work has allowed the monitoring of the mobility of thin tubular projections originating from plastids (Kohler et al., 1997a; Tirlapur et al., 1999) and the movement and shape changes of the nucleus (Chytilova, Macas, and Galbraith, 1999). Furthermore, by generating a protein fusion between GFP and a spliceosomal protein, the dynamics of coiled bodies, nuclear organelles involved in RNA processing, have been observed in higher plants for the first time (Boudonck, Dolan, and Shaw, 1999).

By creating GFP fusions to known cellular proteins or their putative targeting peptides, it has also been possible to determine the dynamics and localization patterns of these proteins in living plant cells. For example, GFP linked to phragmoplastin, a protein known to be associated with cell plate formation, has allowed the dynamics of early cell plate development to be observed in vivo (Gu and Verma, 1997). A unique family of calmodulin-dependent Ca$$^{2+}$$ ATPases were localized to the ER and nuclear envelope of Arabidopsis (Hong et al., 1999) and a calmodulin-binding transporter protein was shown to be associated with the plasma membrane of barley aleurone proplasts (Schuurink et al., 1998). Various proteins (e.g., CRY2, COP1, phyB) implicated in light signal transduction were linked to GFP and shown to translocate to the nucleus (Kleiner et al., 1999; Stacey and von Arnim, 1999; Yamaguchi et al., 1999; Stacey, Hicks, and von Arnim, 1999), while an isocitrate dehydrogenase isoform fused to GFP was demonstrated to localize exclusively to the mitochondria (Galvez et al., 1998). GFP fusions to transit peptides of phage-type organellar RNA polymerases also allowed localization of this protein to the mitochondria and plastids. These data provided additional support for the involvement of these polymerases in the transcriptional machinery of plant mitochondria and plastids (Hedtke et al., 1999). Therefore, with the availability of several spectral variants of GFP and the ability to target GFP to almost any cellular organelle desired, we predict that the direct interactions between these different organelles and/or protein fusions will be an area of intense research.

**Macromolecular transport and virus movements in plants**—Virus movement in plants is a complex process that is generally considered to involve both cell- to-cell movement via the plasmodesmata (Reichel, Mas, and Beachy, 1999) and systemic transport via the vascular system (Nelson and van Bel, 1998). Despite being intensively studied, the pathways and mechanisms by which viruses moved in plants were poorly understood primarily because of an inability to observe the infection process in live tissue over time (Nelson and van Bel, 1998; Santa Cruz, 1999). However, through the observation of GFP fluorescence from chimeric viruses, researchers can effectively monitor the vein classes by which viruses move after infection in near-real time conditions (Fig. 5; Roberts et al., 1997; Cheng et al., 2000).

New insights as to the manner in which viruses move from cell to cell are also beginning to come to light. Although the association of viral components with some of the host cell components has been reported previously, these associations were demonstrated mainly through electron and light microscopy of fixed plant tissues (Nelson and van Bel, 1998). With modified viruses expressing GFP fusions to viral proteins, specific localization of viral proteins to plasmodesmata (Itaya et al., 1997; Oparka et al., 1997) and other subcellular components of the plant host have been elegantly demonstrated. For example, by linking the tobacco mosaic virus movement protein (MP) to GFP, colocalization of MP-GFP to cellular actin was observed and MP was demonstrated to bind actin in vitro (McLean, Zupan, and Zambrayski, 1995). MP-GFP has also been shown to localize to fluorescent aggregates and filamentous structures in infected BY2 cells. Treatment of infected cells with microutubule-depolymerizing compounds disrupted the filamentous strands providing evidence for MP-microtubule association (Heinlein et al., 1995, 1998). These studies all indicate that the local spread of viruses could be facilitated by interactions between a virus and these components of the host plant (Reichel, Mas, and Beachy, 1999).

The progress made from studies on plant virus movement has had a significant impact on plant cell biology and plant physiology. The ability of viruses to penetrate the physical barriers presented by the plant has opened new ideas on the fundamental mechanisms by which macromolecules are transported throughout the plant. These transport stories have revealed perhaps one of the major discoveries of the past decade, that plant nucleic acid can be transported from cell to cell. This was originally reported for the Knotted gene in meristems (Lucas et al., 1995), but there are now an ever-expanding set of results pointing to a remarkable informational macromolecular exchange between plant cells that are likely to coordinate many aspects of plant development and function (Lucas and Wolf, 1999; Oparka et al., 1999). The reader is referred to the several excellent reviews published recently that have thoroughly addressed these issues (Lazarowitz, 1999; Lazarowitz and Beachy, 1999; Santa Cruz, 1999; Thompson and Schulz, 1999).

**Plant cellular signaling**—Another exciting use of GFP fusion proteins is to allow the visualization and quantification of protein–protein interactions. This technique is called fluorescence resonance energy transfer (FRET) and relies on the phenomenon whereby a donor fluorescent molecule, when excited with the appropriate wavelength of light, transfers some of its emission energy to an adjacent acceptor chromophore. Since FRET is dependent on the proximity of the donor to the acceptor, measurement of the efficiency of FRET can be used to estimate the distance between donor and acceptor fluorophores. Therefore if two different proteins or molecules are tagged with either a donor or acceptor fluorophore, FRET can determine whether these proteins are in close association (Gordon et al., 1998).
The concept of FRET in combination with GFP technology offers new methods for elucidating signal transduction pathways in plants. For example, spectral variants of GFP were recently used to construct a novel intracellular Ca\(^{2+}\) sensor referred to as "cameleon." This probe was designed so that two spectral variants of GFP with overlapping emission and excitation spectra were fused at opposite ends of calmodulin (a Ca\(^{2+}\)-binding protein) and the M13 peptide, (the calmodulin-binding domain from myosin light chain kinase). The two varieties of cameleon constructs are the yellow-cameleon, which consists of cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP), and the blue cameleon, which consists of blue fluorescent protein (BFP) and GFP (Miyawaki et al., 1997).

Cameleons report Ca\(^{2+}\) in the following manner. Binding of Ca\(^{2+}\) to the calmodulin within the cameleon induces a conformational change causing it to bind to the M13 peptide. The cameleon molecule is folded in the process such that the donor (e.g., CFP) and acceptor (e.g., YFP) are brought in to closer proximity to each other, thereby increasing FRET efficiency. The donor:acceptor fluorescence ratio can then be calibrated to the cytoplasmic Ca\(^{2+}\) concentration (Miyawaki et al., 1997, 1999). Although the BFP constructs suffer from low signal strength in plants, plant-expressed CFP constructs are bright (Blancaflor, Dowd, and Gilroy, unpublished data) making the CFP-YFP cameleon the appropriate choice for use with plant tissues.

Cameleons were first used to measure intracellular calcium in mammalian cells (Miyawaki et al., 1997; Emmanouilidou et al., 1999), but the yellow cameleon construct has now been successfully engineered into plant cells (Allen et al., 1999; Fricker and Oparka, 1999; Gadella, van der Krog, and Bisseling, 1999). Allen et al. (1999) constitutively expressed this yellow cameleon in Arabidopsis and used this to investigate cytoplasmic Ca\(^{2+}\) changes in guard cells. Extracellular Ca\(^{2+}\) or ABA application induced cytoplasmic Ca\(^{2+}\) transients in guard cells, which were similar to the changes observed using fluorescent dyes. These results indicate that GFP-based Ca\(^{2+}\) indicators are suitable for plants and therefore will be of enormous value in understanding the complex role of Ca\(^{2+}\) in plant cell signal transduction.

In addition to Ca\(^{2+}\), pH has also been shown to tightly regulate numerous cellular signaling events and physiological processes in plant cells (Guern et al., 1991). Like previous studies with Ca\(^{2+}\), measurement of cytoplasmic pH has relied on fluorescent ratiometric dyes (Bibikova et al., 1998; Fricker et al., 1999; Scott and Allen, 1999). GFP-based pH indicators have also been recently developed for monitoring cytoplasmic and organelle pH in mammalian cells (Kneen et al., 1998; Llopis et al., 1998). Various laboratories are attempting to engineer these GFP-based pH indicators into plants, and it will be exciting to see whether these pH sensors function in living plant cells.

The use of GFP-based ion sensors to study plant signal transduction is still in its infancy, but is likely to generate information not previously obtainable with the traditional fluorescent dyes. This is due to the fact that these GFP-based ion sensors can be easily targeted to organelles (see discussion above); therefore the subtle changes in ions that appear to be part of the signaling cascades of some plant processes such as gravitropism can now be determined (Legue et al., 1997; Rosen, Chen, and Masson, 1999; Scott and Allen, 1999). Furthermore, the cameleon indicators can now be imaged with video-rate scanning two-photon microscopy (Fan et al., 1999); therefore it will be possible to quantify rapid changes in Ca\(^{2+}\) deep in an intact plant organ.

**GFP as a cell- and tissue-specific marker**—In developmental biology, labeling of cells to follow cell fate has relied mainly on the microinjection of dyes into specific cell types or immunocytochemical detection of specific cell types after tissue fixation (Brand, 1999). With GFP, it is now possible to mark specific cells or tissues in living Arabidopsis plants and follow their fates directly during development (Fricker and Oparka, 1999; Haseloff, 1999). This was accomplished by using the targeted gene expression approach that uses the GAL4 yeast transcription activator. In this "enhancer-trap" strategy, the GAL4 gene is randomly integrated into the Arabidopsis genome, bringing it under the control of different genomic enhancer sequences that can direct a wide range of expression patterns in the plant. However, in order to obtain efficient expression, it was necessary to alter codon usage by employing a derivative sequence (GAL4-VP19). With GAL4-VP19 linked to mGFP5, which codes for ER-targeted GFP, the generated expression patterns of GFP in Arabidopsis roots could be readily visualized with a confocal microscope (Haseloff, 1999).

Using the enhancer trap approach, root-cap-specific promoters were recently identified and used to genetically ablate (i.e., selectively kill through the localized expression of a cytotoxic protein) root caps of Arabidopsis to study the consequences that such ablations have on root development (Haseloff, 1999). With the availability of specifically marked cell lines, researchers were also able to follow cellular fate and infer positional information that contributes to a specific type of patterning in roots and shoots (Berger et al., 1998a, b; Sabatini et al., 1999). Cell-specific marking using GFP has also been useful in the identification of endodermal protoplasts for patch-clamp experiments (Maathuis et al., 1998) and companion cells for single-cell detection of gene transcripts (Brandt et al., 1999).

One of the more exciting prospects of the enhancer trap approach is the ability to activate other genes of interest in the GFP-marked cell lines. This can be accomplished by subcloning the desired gene behind a tandem array of five optimized GAL4 binding sites (the Upstream Activation Sequences) and transforming plants with this construct. In the absence of the GAL4 transcriptional activator, however, the desired gene is not expressed. By crossing this plant with a line with a known pattern of GAL4 expression, known because of the GFP expression patterns, the silenced gene is activated only in the cells where GAL4 is expressed (Haseloff, 1999). Using this approach, it should be possible, for example, to express the GFP-based cameleon or pH indicators in specific cells or tissues in the plant and monitor signaling-related ion fluxes in these cells. Since the fluorescence signal will be coming exclusively from specific cells, the problem of interfering signals from other tissues is eliminated. Furthermore, it should also be possible to express particular GFP-protein fusions such as the GFP:MAP4 or GFP:talin fusions described above in specific cells and follow the dynamics of the cytoskeleton as these cells progress developmentally.

**OPTICAL TECHNIQUES FOR MICROMANIPULATION OF PLANT CELLS**

In addition to the improved ability to visualize dynamic structures and quantify various physiological processes in
cells, optical microscopy is now being used as a precise tool to manipulate both the physical and chemical properties of the cell. The fields of chemistry and physics have contributed substantially to the development of these new optical approaches and a representative from each field is discussed below.

**Caged probe technology**—Caged or photoactivatable probes provide the opportunity to manipulate cell biochemistry or signaling activities in situ using light. All caged probes operate on a similar theme. A biologically active molecule is derivatized by a caging group, which then sterically blocks the activity of the molecule. The caging group is chosen carefully to be photoactivatable. Thus, when the caging group absorbs light (usually only UV light is energetic enough for these applications), it photolyzes, releasing the biologically active molecule. Some photolysis by-products are also released, and their potential biological activity has to be carefully checked. However, the caged molecules used in plant cells to date have revealed no indication of biological activity or cytotoxicity of these by-products.

When the caged molecule is loaded into the cell (e.g., by microinjection), then illumination using a standard fluorescence microscope is all it takes to release the molecule in the cell. More sophisticated setups use a flash UV illuminator or localized UV laser irradiation to produce highly controlled temporal or spatial uncaging. In addition, the more UV light that is used, the more probe is activated. Thus the cell biologist can now control the spatial, temporal, and amplitude aspects of a molecule’s activity in the living cell. As with fluorescent probes, the range of caged probes that are available has grown dramatically in the last few years. There are now caged hormones, amino acids, peptides, drugs (such as caged taxol), nucleotides, ionophores, fluorochromes, and a range of caged-chelators and even caged-nitric oxide (Haugland, 1999). Most are commercially available, although some of the more specialized reagents must be obtained from the laboratory where they were synthesized.

Not surprisingly this technology has been applied to manipulate a wide range of control features of animal cells (Adams and Tsien, 1993). However, though used in several plant studies, its potential has yet to be fully explored by plant cell biologists. For example, caged Ca²⁺, Ca²⁺ chelators, and ionophores have been used to show a requirement for Ca²⁺ in the response to GA and ABA in aleurone cells (Gilroy, 1996), phytochrome action (Shacklock, Read, and Trewavas, 1992; Fallon, Shacklock, and Trewavas, 1993), the signaling pathway of stomatal closure (Gilroy, Read, and Trewavas, 1990) and in the localization of growth in pollen tubes (Fig. 6; Malho and Trewavas, 1996; Bibikova, Zhigilei, and Gilroy, 1997). Caged inositol triphosphate (IP₃) has revealed a role for an IP₃ mobilizable internal Ca²⁺ pool in the stomatal closure response (Blatt, Thiel, and Trentham, 1990; Gilroy, Read, and Trewavas, 1990) and pollen tube growth (Franklin-Tong, 1999) and caged ABA has been used to localize one site of ABA perception to an intracellular site in the guard cell (Allan et al., 1994). Given the ability to quantitatively regulate the spatial and temporal release of a caged molecule with subcellular precision, we can predict an increasing application of such approaches to probe the spatial and temporal requirements of regulation in the plant cell.

**Laser micromanipulation**—The use of lasers is not only limited to providing a coherent light source for microscopic imaging of cells, but has also been used for the micromanipulation of whole cells and organelles. Laser micromanipulation can be divided into two types. The first type, called laser tweezers or optical trapping, makes use of a continuous-wave, low-power infrared laser beam. The laser beam is focused on an object at the focal plane of the microscope, which results in the refraction of the incident laser beam. The refraction of the laser produces forces that pull the object toward the laser beam, which can be used to hold the object in place. When applied to cells, moving the laser beam can pull organelles or whole cells from one place to another (Berns, 1998; Greulich and Pilarczyk, 1998).

One example of the use of laser tweezers in biology has been to address the problem of gravity sensing in the green alga Chara. Chara is anchored to its substratum via single-cell, root-like structures called rhizoids. The tips of these rhizoids contain numerous vesicles that are filled with heavy mineral deposits (Fig. 7). Upon tilting the rhizoids by 90°, these vesicles sediment rapidly to the new lower flank of the cell. Curvature commences shortly after sedimentation, and it is believed that the sedimentation of these vesicles is responsible for the initial perception of gravity leading to the redirection of rhizoid growth (Sievers, Buchen and Hodick, 1996). Laser tweezers have been used to mimic the gravitational displacement of these vesicles and have been shown to alter the direction of rhizoid growth (Fig. 7; Leitz, Schnepp, and Greulich, 1995). By combining laser trapping experiments and imaging...
concludes and prospects
The rapid advances in optical probes and imaging technology make these exciting times for plant cell biology. The three lines of technological advances that have brought us to this unprecedented view of the dynamic plant cell show no sign of slowing. First we can predict a continuing impact of ever-increasing computing power. Image analysis is inherently a computer-based science and as computing power increases we can predict that techniques to improve resolution and extract even more complex information from raw fluorescence images, such as real-time image deconvolution, will become commonplace. The second thread of advance will be new and exciting imaging technologies. We have highlighted multiphoton microscopy as a novel imaging approach with enormous promise, but this is but one of the many technologies, such as fluorescence lifetime imaging and correlation imaging, on the horizon. The third line of advance will be in fluorescent and luminescent probes. GFP technology is clearly an important step towards the ultimate goal of noninvasively monitoring the dynamics of the components of the plant cell, but there will be an increasing need for fluorescent probes to monitor not only levels and distribution of regulators but also their activity. For example, recently, calmodulin activity has been imaged using novel fluorescent dyes (Hahn, Waggoner, and Taylor, 1990; Torok et al., 1998) and a GFP-based probe (Persechini and Cronk, 1999). These successes suggest that developing probes to image the activities of cellular regulators is an attainable goal.

The most significant advances will be made by combining these three areas of technology. For example, fluorescence correlation microscopy uses powerful computational analysis of fluorescence detected by very sensitive imaging sensors (Haupts et al., 1998; Schwille et al., 1999). It has been used to look at the changes in parameters such as molecular diffusion constants of GFP fusion proteins to image their interactions with other cell components (Brock et al., 1999; Trier et al., 1999). Computation, imaging, and fluorescent probes have come together to reveal the dynamics of the environment around a single molecule and have provided a very powerful tool to monitor the activities of the living cell. The application of such technologies to the vast array of unanswered questions in plant development, function, and regulation will be a major goal for plant cell biology researchers in the new millennium.

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