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The illuminated plant cell



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The illuminated plant cell

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The past decade has provided biologists with a palette of genetically encoded, multicolored fluorescent proteins. The living plant cell turned into a 'coloring book' and today, nearly every text-book organelle has been highlighted in scintillating fluorescent colors. This review provides a concise listing of the earliest representative fluorescent-protein probes used to highlight various targets within the plant cell, and introduces the idea of using the numerous multicolor, subcellular probes for the development of an early intracellular response profile of plants.

Visualizing the plant-cell interior

The wall encasing the plant cell has been the largest barrier to the visualization and understanding of subcellular processes in living plants. Traditionally, plant scientists have relied on squashing, maceration, sectioning or enzyme-mediated degradation of the cell wall to gain access to the inner compartments of the plant cell. Although detailed observations of the plant cell constitute the foundations of plant biology, the fact remains that many of the descriptions are extrapolations of observations made on fixed, dead plant tissue. By comparison, the analysis of live-cell phenomena, such as cytoplasmic streaming and organelle interactions, has been rather limited ([1] and references cited therein) because only those plant tissues that allow light to be transmitted through are amenable to non-invasive visualization techniques involving time-lapse video recordings. With the advent of fluorescence microscopy and the availability of cell permeant dyes, such as the nucleotide-binding SYTO stains, the endomembrane-staining DIOC6 and FM4-64 dves and the mitochondria-specific mitotracker dves [2]. plant biologists have obtained a short time window for live-cell imaging before toxicity-related concerns become pertinent. Also, microinjection of specific stains and fluorescent-protein analogs into living plant cells has become a powerful tool for the observation of subcellular processes [3]. Unfortunately, microinjection procedures do require skilled researchers, are often labor intensive and are limited in terms of useful cell types, observable cell numbers and experimental reproducibility. Thus, only a handful of researchers could explore their potential and, for plant biologists, they never reached the status of a routine technique.

Here, I provide a brief overview of how our ability to look inside the plant cell received a tremendous boost in the early 1990s, with the cloning and rapid availability of a 27-kDa green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* [4].

Fluorescent proteins light up the plant cell interior

The genetically encoded GFP swept away many of the cell wall-imposed limitations on live imaging of the plant cell interior because, in stark contrast to the cumbersome external loading of stains and dyes, GFP and its derivative fluorescent proteins (FPs) are produced by the cells themselves and do not require exogenous substrates, cofactors or chemical treatments for their activity [5,6]. Through their fusion to specific nucleotide sequences, FPs can be targeted to literally any compartment or component of the cell. Once introduced into a plant cell, either for transient or for stable transgene expression after integration in the plant genome, FPs follow the general rules governing subcellular protein dynamics, localization and interactions. The fusion proteins are thus able to respond to both cell-intrinsic and external environmental cues. Through concomitant advancements in non-invasive, CCD-based epifluorescent and confocal laser scanning microscopy. FPs can be readily visualized in living plant cells [6].

Whereas, many modern laboratories using FPs in their research trace their initial acquisition of GFP clones to Douglas Prasher and Martin Chalfie [4], Roger Tsien [7] or to the commercial source Clontech (http://www.clontech. com), a large portion of the credit for modifying GFP for optimal expression in plants and popularizing its use among fellow plant scientists through its unconditional sharing goes to Jim Haseloff and his research team [8]. By late 1997, many plant research laboratories had introduced the cDNAs for mGFP5 and mGFP5-ER (targeted to the endoplasmic reticulum) into their plant dissection strategies involving transcriptional and translational fusion proteins.

The rapid acceptance of GFP as a live reporter protein provided a strong motivation for the creation of newer versions of FPs with altered spectral characteristics [9]. Today, multicolored FPs spanning the visible spectrum have been obtained from a variety of organisms [9,10]. In plants, two complementary FP-based investigative strategies have been adopted:

- (i) those creating chimeric translational and transcriptional constructs using FPs to gain spatiotemporal information about gene activity in the plant developmental context;
- (ii) those that specifically target FPs to organelles and vesicles or to the cell boundary components to understand subcellular dynamics and interactions (Table 1).

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		GFP and C-terminal vacuolar sorting signal (amino acid residues 318–324) fusion construct	10.11

^aThe late-type endosome is also referred to as a prevacuolar compartment or multivesicular body [31].

^bThe apoplastic space and the wall are not considered subcellular components but are intimately related to the plant cell.

Whereas this review focuses largely on subcellular markers for use in plants, the visualization of FPs within a tissue/organ has been pivotal in educating us about various important aspects of plant development including those related to signaling [11,12] and patterning [13,14]. Many of the stable transgenic lines of *Arabidopsis thaliana* exhibiting tissue- or cell-type-specific expression patterns, such as the GAL4–GFP enhancer trap lines from Jim Haseloff and from Scott Poethig, have been created using the mGFP5-ER construct and are now available through public domain seed stock centers, such as NASC (http://arabidopsis.info/).

In addition to their uses as marker lines in research and in helping to elucidate gene function within the developmental context, *Arabidopsis* lines exhibiting tissue-specific GFP expression are turning out to be excellent teaching material.

Understanding subcellular dynamics and interactions through targeted FPs

The ss-GFP-HDEL fusion construct, one of the first subcellular targeted probes to be created, fluorescently

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Review



Figure 1. Diagrammatic representation of a generalized plant cell providing the reference locations of subcellular components and compartments to which the fluorescent proteins are targeted, along with the pertinent citations. The diagram is not drawn to scale and does not accurately reflect the relative numbers of subcellular components. Ribosomes [63] and proteasomes [64] have nuclear as well as free cytoplasmic locations. The figure is based on Ref. [62].

highlights the endoplasmic reticulum (ER) [8] and has been used by many laboratories to obtain their first glimpse of green fluorescence in plant cells. Subsequently, this probe has been used as a useful control when targeting FPs to other organelles (Figure 1). Although every discovery relating to the inner workings of the plant cell should be considered important and find a mention in this overview, a listing of the myriad of discoveries and the numerous probes created during the past decade has to be curtailed owing to space limitations. However, in recognition of the rapid growth of the field and the necessity of keeping it frequently updated, a new online resource devoted to 'the Illuminated Plant Cell' (http://www. illuminatedcell.com/) is being created. This website aims to provide a comprehensive listing of probes and related information to the community. Nevertheless, certain findings, such as the elucidation of mechanisms of plasmodesmatal functioning and viral movement [15], the rediscovery of plastid stromules [16], the visualization of

the intricate cytoskeletal organization [17,18] and unraveling of its functioning [19–22], the recognition of novel actin-based mechanisms for organelle motility [23–25] and subcellular interactions [26–28], the recognition of exocytosis- [29] and endocytosis-mediated [30,31] mechanisms in plant development, the visualization of cellulase synthase (CESA6) organization and *in situ* activity [32], and the use of targeted GFP in microscope-based mutant screens [33–35], have resulted directly from the use of targeted fluorescent proteins.

One of the benefits of using FPS for live imaging is the observation of transient subcellular phenomena, such as the conditional highlighting of spindle-shaped ER bodies [8] in response to defense-inducing conditions (e.g. herbivory [36]) and the quick evaluation of organelle behavior in response to stress and apoptotic signals. Conditional dual targeting of certain probes, such as the ERD2–GFP, which can accumulate specifically in Golgi bodies (Figure 1) or be localized in both the ER and Golgi stacks [23], has also been possible using targeted FPs. Furthermore, although (based on similar localization patterns) a large number of proteins are known to highlight peroxisomes and mitochondria [37,38], the need for multiple probes has been felt for the labeling of F-actin [17,39] and microtubules [18,40] following concerns that a single probe might not label the entire gamut of arrays displayed by these ubiquitous cytoskeletal elements. Thus, the number of targeted probes created sometimes out of dire necessity but often through routine experiments designed to localize a gene product, continues to grow steadily. An approach that has been particularly useful in generating probes has been the creation of random cDNA::FP fusions to identify new subcellular structures in plant cells [37,41–43]. The increased diversity in probes targeted towards the same subcellular structure provide the researcher with a range of protein tools to suit specific experimental requirements, act as the much-needed independent probes for controls and confirmation of observations, and are leading to more detailed dissection of suborganellar properties [21,22,25,44]. Because the creation, merits, demerits and numerous uses of probes targeted to specific organelles have been reviewed in detail [44-47], Table 1 and Figure 1 do not provide a comprehensive listing of every targeted fusion protein created to-date. Rather, they serve to emphasize the fact that a vast majority of subcellular compartments and components of the plant cell have become fluorescently highlighted.

The following sections briefly explore the long-term implication of the availability of multiple subcellular probes for achieving a better understanding of the plant cell.

A target within reach: the multicolored plant cell

Most early organelle-targeted probes used different versions of GFP. These probes allowed the clear visualization of the targeted organelle (Table 1) and the dissection of interactions occurring between similar organelles, such as chloroplast-chloroplast interactions through stromules [16] or the 'kiss and run' transient interaction between mitochondria [45]. However, they did not allow the visualization of interactions occurring between two or more different organelles. Single FP-based observations thus reveal only a small part of the dynamic subcellular world. The vital subcellular cooperation and coordination of interactions can best be pursued when more than one FP tags are used for targeting different structures [24,27,48]. Therefore, there is a growing trend to replace GFP with other compatible, colored FPs to achieve the simultaneous, multicolor visualization of multiple organelles and their interactions. For example, the GFP-MAP4 (MBD) probe, which has served as a very useful label for cortical microtubule arrays [18], is now also available in cyan (CFP-MAP4) [48], yellow (YFP-MAP4) [27] and red (DsRed-MAP4) [49] versions. The availability of these different FP-tagged versions of MAP4 has allowed the fine analysis of microtubule dynamics and led to numerous new insights [19,20,26,27,48,49]. The multicolor visualization approach is also resulting in a slow reversal of the tendency to exclude chlorophyll autofluorescence in green tissues by creating specific narrow band filters for GFP. In fact, in many live visualization strategies, not only does the orange-red chlorophyll provide a bright counter-fluorescence to GFP but it also allows the simultaneous visualization of chloroplasts with other FP-labeled organelles (Figure 2a,h). Most importantly, the photobleaching of chloroplasts can be used as an internal control for photodamage-induced artifacts (Box 1). Figure 2 provides a few examples of multicolor probes that are being used for the simultaneous visualization of different organelles in plant cells. In the simplest strategy for creating a multicolor line, two stable transgenic lines carrying dissimilar FP-probes targeted to separate organelles can be crossed. By including chloroplasts as autofluorescent structures, this strategy easily allows the visualization of three different organelles within the same cell (Figure 2a,h,i). Although subject to limitations imposed by gene-silencing mechanisms, the two-color-FP-containing plant can be further transformed and selected for inclusion of an additional FP-marker (Figure 2b-e). Although the expression of four FPs [e.g. ECFP (emission max. ca. 475 nm), EGFP (emission max. 509 nm), EYFP (emission max. ca. 527 nm) and RFP (monomeric DsRed/mRFP, emission max. ca. 607 nm) [9,10,50]] can be achieved, the actual visualization and convincing separation of more than four colors within a cell is still a technically challenging task. Theoretically, very fine spectral separation can be achieved by confining data collection to peak emission wavelengths for different

Box 1. Knowing the 'darker side' of FP-based technology

The FP-based method for studying plants does require a cautionary note. Because each FP has a specific size and characteristic folding properties, the addition of an FP-tag to determine the subcellular localization and behavioral properties of another protein of interest must be mindful of issues related to alterations of protein mobility, turnover and stability, in addition to the possible alterations in subcellular localization patterns. The size of the protein being fused to the FP, the information related to the folding of the fusion protein, the shielding of a signal sequence or the inadvertent snipping off of a portion of the C-terminal sequence, the introduction of a 'hinge' between the protein of interest and the FP, the unintentional introduction of a mutation in a PCR-based cloning approach, are all considerations that should be matched by adequate controls. Artifacts might also result from using multimeric versus monomeric versions of a given FP or by relying overly on transient overexpression data versus stable expression (or vice versa) for a particular fusion-protein probe. Although transient expression experiments, including those resulting in a sudden subcellular flooding of a FP-probe through the use of inducible promoters, can result in major misinterpretations of protein behavior and localization, even stable transgenic lines should be carefully screened for the range of protein expression, plasmid insertion related effects and the possible developmental consequences before putting forward an opinion on gene function. The most common misinterpretations result from faulty imaging methods and conditions, especially where broadband filters that allow a bleed-through of native autofluorescence are used. This specific imaging artifact is a major concern in conclusions based on FP-colocalization or FRET interactions. Great caution must be exercised when observing motile organelles over time, because even transient changes in high-intensity laser-induced photobleaching as well as membrane damage can greatly skew motility data. Again, data overextraction through the use of non-transparent algorithms and data extrapolation software are concerns associated with FP-technology. More detailed discussions on the advantages and disadvantages of FPbased probes and the limitations of light microscopy are available [5-7,9,10,47,50,60].

Review



TRENDS in Plant Science

Figure 2. Transgenic lines in *Arabidopsis thaliana* carrying combinations of probes targeted to different organelles and compartments are being created for simultaneous multicolor visualization of the living plant cell. (a) Chloroplasts (ch; red autofluorescence), mitochondria (mito; green fluorescent) and peroxisomes (per; yellow fluorescence [24]) visualized simultaneously. (b) Peroxisomes (per; false allocated red color for YFP–SKL target [24]) and endoplasmic reticulum (GFP targeted to the ER [8]) visualized in a single confocal section. (c) Golgi bodies (Gol; false allocated red color for ERD2–GFP target [23]) and actin microfilaments (Af; false allocated green color for YFP–SKL target [17]) visualized in a pavement cell. (d) Simultaneous visualization of the two major cytoskeletal elements in plants through GFP labeled F-actin (Af; targeted through GFP–mTalin [17]) and DsRed2–MAP4(MBD)-labeled microtubules (Mt; red color). (e) Visualization of YFP-labeled plasma membrane [37] and GFP-labeled microtubules [18]. (f) Peroxisomes (per; YFP–SKL target [24]) and cyan colored F-actin (Af; CFP–mTalin) covisualized using dual band CFP–YFP filter (Chroma technology-filter set 59017). (g) Nucleus (Nu) highlighted using GFP–NLS [65] within a microtubule cage (Mt; red color; MBD). (h) Multicolor visualization can offer clues about the relative amounts of proteins, viz, GFP (green) and Chlorophyll (red autofluorescence) as well as their coincident localization (yellow) in different chloroplasts (ch) within the same area of the cell. Also shown are stromules (st) connecting chloroplasts. (i) The simultaneous visualization strategy can be greatly augmented by using morphological criterion in addition to multicolor FP targeting as shown in this single confocal scan. The round GFP–NLS target [65] nucleus (Nu), is clearly distinguished from the GFP–mTalin [17] targeted F-actin (Af) and the net-like endoplasmic reticulum (ER; targeted using a HKDEL [8] fusion to DsRed2).

probes. Whereas this is possible for brightly fluorescent cells where the different FPs display roughly similar levels of fluorescence intensities, the large overlaps in excitation and emission spectra for most commonly used FPs, combined with the subcellular motility of organelles, frequently create confusing color-overlaps. Approaches aimed at increasing wavelength resolution for multicolor imaging range from spectrophotometric separation or/and algorithm-based FP-specific spectral profiling for protein discrimination. Alternatively, FPs possessing more stringent spectral characteristics are available for use [9,10]. Recently, the creation of several inducible promoters has introduced another exciting range of possibilities whereby the FP remains unexpressed until the chimeric gene is triggered by the exogenous application of an inducing chemical or change of temperature regime [21,51,52]. In addition, several photo-inducible FPs have become available. These include PA-GFP (photoactivable-GFP), which becomes activated by 405 nm-light to produce a many-fold increase in fluorescence [53]. PA-GFP has been targeted to the ER to understand protein dynamics within this compartment [28]. Similarly EosFP [54] and Kaede [55], two FPs that rapidly change color from green to red upon activation by near-UV light (ca. 390 nm) have been used for the visualization of endocytosis events at the plasma membrane [30] and to improve our understanding of mitochondrial fusion and division [56], respectively. FPs that accumulate in the cytosol and respond to specific activator molecules, such as reactive oxygen species [57] or to changes in the status of various ions, including H⁺, Ca^{2+} , Cl^{-} and NO_{3}^{-} (reviewed in Ref. [58), and destabilized versions of FP [59] have been developed and are valuable additions to the fluorescent protein tool kit.

Although not discussed here, advanced imaging techniques, such as bimolecular fluorescence complementation (BiFC [60]), biluminescence resonance energy transfer (BRET), Forster or fluorescence resonance energy transfer (FRET), fluorescence lifetime imaging (FLIM) and fluorescence recovery after photobleaching (FRAP [61]) (reviewed in Refs [5–7,9,10,50]), are all off-shoots of FP-based technology whose application in plant research is gathering momentum.

Targeted FPs and EIRPing of plants: an emerging concept

As discussed earlier, FPs have provided us with the ability to look inside living plant cells and have revealed that, within their rigid walls, plant cells actually display rapid subcellular dynamics. To ensure survival, a rooted plant needs to respond very quickly to diverse environmental cues. Interestingly, our understanding of a plant's response to a given stimulus comes from observations that are usually made long after the causal event has occurred. For example, although we know that, like all other living organisms, plants suffer from stress, we do not know the earliest subcellular indications of stress shown by a plant cell. Similarly, we recognize that plants are susceptible to pests and diseases but have only a hazy idea about the earliest responses of a plant cell to the invasion of its epidermal surface.

The fluorescently illuminated plant cell thus has a lot of new information to offer through its rapid response to environmental cues. In the long term, this information can be judiciously combined with molecular-genetic strategies to devise better strategies for plant improvement and management. These thoughts and the availability of numerous targeted fluorescent protein probes and transgenic lines have led to the idea of generating an early intracellular response profile for plants (EIRPP; http:// www.uoguelph.ca/~jmathur/research/EIRP.html). Proof of concept studies for EIRPP using the model plant *Arabidopsis thaliana* are already underway.

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An overview on FP-based contributions to understanding plants merits the inclusion of many more references than has been possible here. I hope that the comprehensive online resource being created will serve as a suitable atonement for the omissions. I thank Alison Sinclair and Preetinder Dhanoa for help in compiling the initial list of FP probes that appeared in a similar mini-review [62], Anshudeep Mathur for the creation of the Interactive flash animation and the design of the webpage, David Logan, Sean Cutler, Geoff Wasteneys, Rob Mullen and Hugo Zhang for their critical inputs. The Natural Sciences and Engineering Research Council of Canada (NSERC) fund the EIRPP program initiated by my laboratory.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants. 2007.08.017.

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