Fluorescent Proteins in Transgenic Plants

Reginald J. Millwood, Hong S. Moon, and C. Neal Stewart Jr.

Abstract Fluorescent proteins (FPs) have revolutionized many areas of biological research. In particular, plant biotechnology has been significantly advanced by harnessing the power of FPs. *Aequorea victoria,* green fluorescent protein (GFP), has been the most studied of the proteins, but many new FPs are discovered each year. We provide here a timely review of the current uses of FPs in whole plants and we look at the color palette of candidate proteins. Lastly, we discuss current instrumentation and methods for detection and quantification of FPs in plants.

1 Introduction

For nearly two decades, fluorescent proteins (FPs) have been invaluable tools in basic and applied scientific research. However, FPs are not new to science. In fact, they have been studied for more than 50 years. The most widely studied FP is the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria*. GFP was first isolated by Osamu Shimomura at the Friday Harbor Laboratories, Washington, USA [1]. Shimomura was not interested in GFP as a biotechnology tool. Rather, his research interest was to understand the chemistry and biochemistry of *A. victoria's* bioluminescence. It was not until Doug Prasher et al. [2] cloned the *gfp* gene that the utility of GFP was fully realized, which enabled the award of the Nobel Prize in Chemistry (2008) to three scientists (Osamu Shimomura, Martin Chalfie, and Roger Tsien), but ironically, not to Doug Prasher. For the first time researchers had accessibility to a marker gene that was useful in vivo, could be detected in real-time, and required no substrates or cofactors. Subsequently, the gene was expressed in *Escherichia coli* and *Caenorhabditis elegans* [3]. Shortly thereafter, the *gfp* gene was subjected to mutagenesis and several new variants

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were created ranging from blue to yellow [4, 5]. These events marked the beginning of what has often been called the "Green Revolution" of biotechnology. The foundation built with GFP research has allowed others to search for new FPs in other organisms [6–12]. At present, scientists have a wide range of FPs to choose from that work for many applications. Here we discuss a brief history of FPs, candidate FPs for plant expression, and FP applications in whole plants. Also included is a description of current instrumentation and methodologies of FP detection.

2 FPs in Model Organisms

Because of established transformation protocols, model organisms were the first to be transformed with GFP. *E. coli* and *C. elegans* [3] were followed closely by fruitfly (*Drosophila melanogaster*) [13] and subsequently mammalian cell lines [14] and yeast (*Saccharomyces cerevisiae*) [15] . Larger organisms, such as tobacco (*Nicotiana tabacum*) [16] , mouse (*Mus musculus*) [17], zebrafish (*Danio rerio*) [18], frog (*Xenopus laevis*) [19], rhesus monkey (*Macaca mulatta*) [20], and pig (*Sus domestica*) [21] were transformed with GFP variants yielding visible green fluorescence. In addition to the many GFP organisms, a red FP (DsRed2) from the coral *Discosoma* sp. has been expressed in the domestic cat (*Felis catus*) [22].

The utility of FPs was quickly realized for applications outside scientific research. Two transgenic organisms in this area are particularly intriguing, the first of which is a commercial ornamental transgenic organism, the "GloFish®" (www.glofish.com), marketed by Yorktown Technologies (Fig. 1a). Under the control of a strong muscle-specific (mylz2) promoter, transgenic zebrafish were transformed with the green fluorescent protein (EGFP) and a red fluorescent protein (dsRed) [23]. GloFish appear brightly fluorescent green, red, or orange (GFP+RFP) when in the presence of an ultraviolet (UVA) aquarium lamp. The original intention for creating transgenic zebrafish was to use them as biosensors for pollutants. For example, the fish appear normal when no pollutants are present, but after pollutant exposure the fish would express an FP to give a visual signal. Zebrafish biosensor research is ongoing, but none have been deployed to date. However, the commercial value was immediately realized when their brilliant fluorescence was observed in the presence of a UVA lamp; these fish are sold to people interested in hobby aquaria. The second and most stunning of the transgenic FP organisms, "Alba," the GFP bunny commissioned by the artist Eduardo Kac, was the central piece of the Eighth Day art exhibit (www.ekac.org/gfpbunny.html#gfpbunnyanchor). Alba, an albino rabbit, was transformed to express GFP and yield a remarkable fluorescent phenotype (Fig. 1b). This caused quite a stir because typically transgenic organisms are not created as works of art but to answer scientific questions. Alba added fuel to the ongoing moral and ethical debate over transgenic organisms. Nevertheless, GloFish® and Alba have exposed the lighter side of science and hopefully they add to greater acceptance of genetically modified organisms.

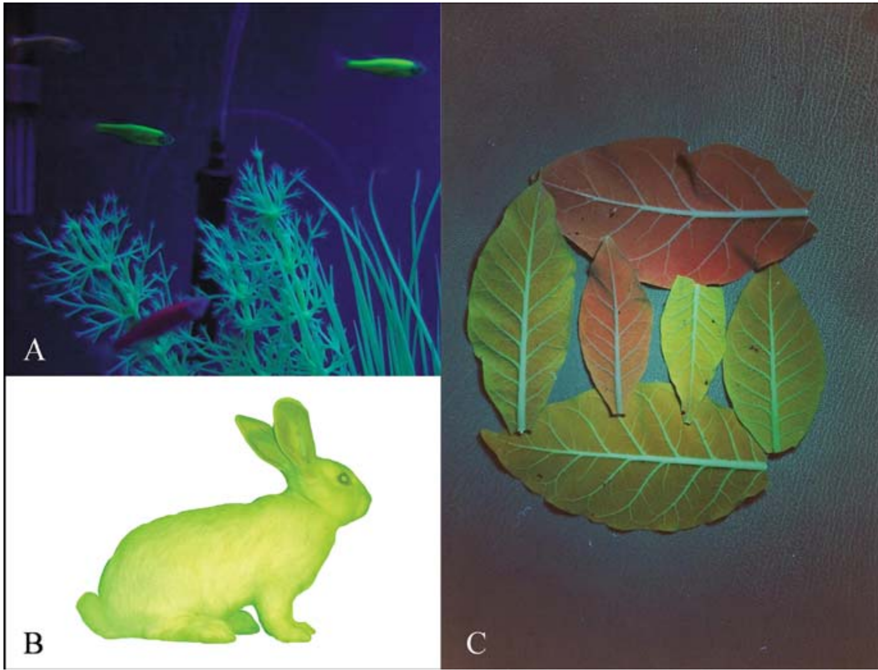

Fig. 1 Transgenic fluorescent organisms. **A** Glofish®, expressing GFP, RFP, and GFP + RFP, marketed by Yorktown Technologies, L.P. **B** Eduardo Kac, GFP Bunny, 2000, transgenic artwork. Alba, the fluorescent rabbit. **C** Tobacco leaves expressing mGFP4. The top leaf is wild type and all others are expressing varying amounts of the protein

3 GFP in Transgenic Plants

Plant scientists were excited by the success of GFP in other organisms. However, they were met with disappointment because wild-type GFP expression was found to be variable in plants. Wild-type GFP expression was first confirmed in plant cells and not in intact plant tissues. GFP fluorescence was observed in sweet orange [24] and maize (*Zea mays*) [25, 26] protoplasts. Transient expression in intact Arabidopsis *thaliana* (Arabidopsis) roots and leaves [26] was also observed but not in Arabidopsis protoplasts [25]. Additionally, stable transformation was confirmed in Arabidopsis but no fluorescence could be detected [27]. With limited success in GFP expression, researchers realized that substantial improvements needed to be made to the wild-type gene.

4 GFP Variants for Plant Expression

Much of the credit for stable GFP expression in plants goes to Jim Haseloff. Haseloff et al. [28] reported that aberrant splicing of wild-type *gfp* mRNA occurred in plant

cells due to a cryptic intron between nucleotides 380 and 463. In Arabidopsis, this 84 nucleotide deletion resulted in a truncated, non-fluorescing protein. Silent mutations were introduced into the splice recognition sites to remove the intron. Two promising variants were produced; mGFP4 and mGFP5 [29]. Expression of mGFP4 was observed in soybean (*Glycine max*) cells [30], Arabidopsis [28], tobacco [31], and other plants. However, mGFP4 did not exhibit stable fluorescence under field conditions even as the protein was expressed in the plant at levels that should have yielded visible green fluorescence [31–33] (Fig. 1c). In contrast, the mGFP5 variant with an endoplasmic reticulum (ER) targeting peptide (mGFP5-ER) [28, 29] showed improved levels of fluorescence, improved thermostability, and dual excitation in UV (395 nm) and blue light (473 nm) of almost equal amplitude. Subsequently, under field conditions mGFP5-ER was found to be expressed twice as much as mGFP4 with higher levels of fluorescence [33].

In plant biology, the S65T mutant contained the most significant chromophore modification to GFP. This change created a single blue excitation peak (489 nm optimum) and slight red shift excitation maxima from 507 to 511 nm [34]. Codon optimization was subsequently performed and up to a 100-fold increase in fluorescence was observed in plant cells [16]. There are two S65T variants that have been widely used in plant science. Haas et al. [35] created sGFP which is a synthetic *S65T* gene with the cryptic intron removed. The other widely used S65T GFP variant is the commercially available EGFP (Clontech). EGFP includes S65T, as well as the F64L and the Y145F mutations and is human codon-optimized [36].

Many GFP variants were made, but not all mutations improved expression levels or brightness. Researchers wanted colors other than green and shortly after the cloning of GFP, blue [4] and cyan [5] variants were produced. Until recently no GFP variant has been produced with an emissions maxima exceeding 529 nm. Mishin et al. [37] reported the first GFP mutant to form a red chromophore. However, only 1–3% of the protein produced matures to the red-emitting state.

5 Other Colors, Other Organisms

With the lack of colors (emission) beyond 529 nm, researchers began searching for other marine organisms that possess GFP homologues. Mikhail Matz et al. [6] were able to clone red fluorescent proteins from non-bioluminescent Anthozoa species, most notably, DsRed [38], which has a number of commercially available variants (Clontech). The Anthozoa FPs have become widely utilized because they are spectrally diverse, ranging from blue to far red. Mutagenesis has been performed on these which has lead to stable, bright, and a few monomerized FPs; most of which have not been tested in plants [39, 40] (Fig. 2). Additionally, Matz and colleagues have recently discovered and characterized many new proteins from corals [12]. Table 1 contains a list of the most promising FPs available to researchers, but discussions on partially characterized FPs (those without published brightness information) are not included.

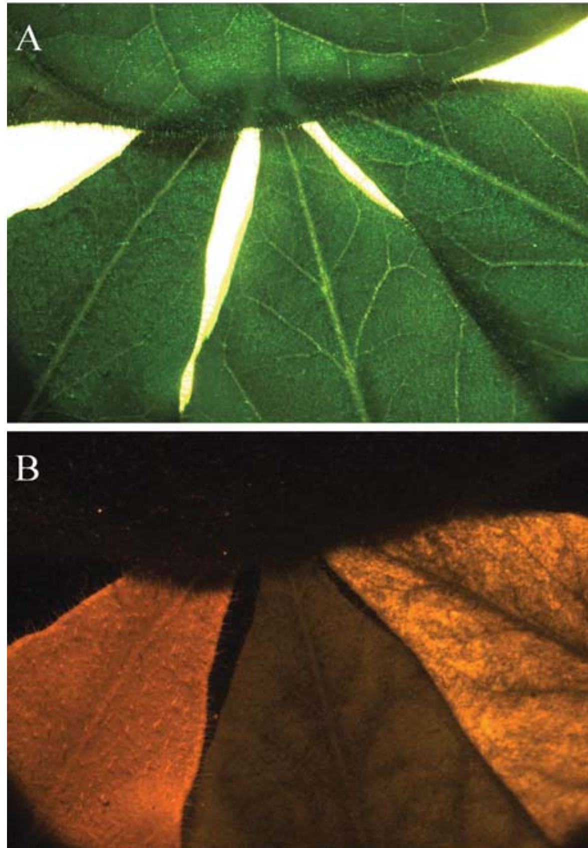

Fig. 2 Examples of FPs expressed in plants. The images were taken with an epifluorescent stereoscope with 90 s exposure, 530/40 nm excitation light and 600/50 band-pass emission filter. The top leaf is wild type and the lower three are transgenic. **A** White light photo. **B** From left to right pporRFP, mOrange, and tdTomato

6 FP Toxicity and Allergenicity

When a novel protein is introduced into an organism, toxicity is a concern and it has been suggested that GFP is cytotoxic to plant cells [27, 28, 41]. The argument was made that GFP fluorescence caused a photonic disturbance that created free radicals and eventual oxidative damage. However, many researchers failed to observe this toxicity in plants that were clearly expressing high levels of GFP [16, 32, 42, 43–46], but see Liu et al. [47] who showed GFP toxicity to mammalian cells. To address the toxicity issue at the whole plant level, Harper et al. [33] tested tobacco in the field using three different GFP variants (mGFP4, mGFP5-ER, and sGFP). Over two growing seasons, seed yield and biomass were recorded. In this study, there was no cost to yield or biomass; therefore, it was concluded GFP is not toxic to plant cells. Many organisms have been transformed with FPs and these show no measurable host cost. It is well documented that plants have many characteristics to deal with excess light that could be damaging to cells.

Table 1 Properties of potentially useful FPs in plants with published extinction coefficients and quantum yields. Many of these have not been tested in plants

	Host Species	Excitation max. (nm) (Extinction coefficient)	Emission max. (nm) (% quantum yield)	Oligomerization (M = monomer, D = dimer, T = tetramer)	Refs
CYAN					
AmCyan	*Anemonia majano*	458 (40)	486 (24)	T	[56]
mmilCFP	*Montipora millepora*	404 (90)	492 (43)	Unknown	[12]
anobCFP2	*Acropora nobilis*	477 (86)	495 (28)	Unknown	[12]
MiCy	*Acropara sp.*	472 (27)	495 (90)	D	[71]
GREEN					
pplu GFP2	*Pontellina plumata*	482 (70)	502 (60)	M	[72]
pmeaGFP2	*Pontella meadi*	487 (98)	502 (72)	Unknown	[72]
pmeaGFP1	*Pontella meadi*	489 (99)	504 (74)	Unknown	[72]
wtGFP	*Aequorea victoria*	395 (27)	504 (79)	M	[64]
mAG	*Galaxeidae sp.*	492 (72)	505 (81)	M	[67]
Azami Green (AG)	*Galaxeidae sp. Azumi*	492 (72)	505 (67)	T	[67]
ZsGreen	*Zoanthus sp.*	497 (36)	506 (63)	M	[56]
pporGFP	*Porites porites*	495 (54)	507 (98)	Unknown	[12]
EGFP	*Aequorea victoria*	488 (56)	508 (60)	M	[64]
GFP Emerald	*Aequorea victoria*	487 (58)	509 (68)	M	[64]
GFP S65T	*Aequorea victoria*	489 (55)	510 (64)	M	[64]
cmFP512	*Cerianthus membranaceus*	503 (59)	512 (66)	T	[68]
plamGFP	*Platygira lamellina*	502 (96)	514 (99)	Unknown	[12]
Kaede	*Trachyphyllia geoffroyi*	508 (99)	518 (80)	T	[70]
eechGFP3	*Echinophyllia echinata*	512 (45)	524 (120)	Unknown	[12]
GFP YFP Topaz	*Aequorea victoria*	514 (94)	527 (60)	M	[64]
GFP YFP Venus	*Aequorea victoria*	515 (92)	528 (57)	M	[65]
phiYFP	*Phialidium sp.*	525 (115)	537 (60)	D	[72]
RED					
mKO	*Fungia concinna*	548 (52)	559 (60)	M	[71]
mOrange	*Discosoma sp.*	548 (71)	562 (69)	M	[62]
tdTomato	*Discosoma sp.*	554 (138)	581 (69)	D (tandem)	[62]
DsRed	*Discosoma sp.*	558 (75)	583 (79)	T	[62]
amilRFP	*Acropora millepora*	560 (49)	593 (91)	Unknown	[12]
pporRFP	*Porites porites*	578 (54)	595 (95)	Unknown	[12]
mStrawberry	*Discosoma sp.*	574 (90)	596 (29)	M	[62]
mCherry	*Discosoma sp.*	587 (72)	610 (22)	M	[62]
eqFP611	*Entacmaea quadricolor*	559 (78)	611 (45)	T	[69]
t-HcRed1	*Heteractis crispa*	590 (160)	637 (4)	T	[66]
mPlum	*Discosoma sp.*	590 (41)	649 (10)	M	[63]
DUAL COLOR					
EosFP	*Lobophyllia hemprichii*	506 (72) 571 (41)	516 (70) 581 (55)	T	[73]
d2EosFP	*Lobophyllia hemprichii*	506 (84) 569 (33)	516 (66) 581 (60)	D	[73]
mEosFP	*Lobophyllia hemprichii*	505 (67) 569 (37)	516 (64) 581 (66)	M	[73]

Additionally, when a novel protein is introduced into a plant that is intended for use as food or feed, human and animal health issues should be addressed. Before any crop expressing GFP could be deregulated, this issue would have to be probed extensively. However, there is evidence that GFP is neutral with regard to oral toxicity and allergenicity. Richards et al. [48] fed purified GFP and pelletized feed made from GFP expressing canola (*Brassica napus*) to rats. At physiological relevant levels GFP was completely digested and there were no allergenic features associated with the protein. However, when rats were fed amounts exceeding physiological levels (1 mg/day purified GFP) it altered the spectral properties of their feces – yielding green fluorescent poop (GFP). Aside from this novelty, the protein had no measurable effects on growth. These findings suggest that GFP and GFP-like proteins are likely safe with regards to oral toxicity and allergenicity.

7 FPs in Plant Research

FPs have become integral tools in developmental biology and functional genomics. They have been fused to numerous proteins to monitor subcellular localization and to tag subcellular structures [49]. Included in a review by Mathur [49] is a useful list of targeted FP probes available for plants. Furthermore, in functional genomics research FPs have been used to assay promoter activity and to clone regulatory elements (as reviewed in Ayalew [50]). Several novel promoters have been characterized by expressed fluorescence. For example, the *taro bacilliform virus* promoter has been characterized by GFP fluorescence in banana and tobacco [51]. GFP has also been used to study RNA interference in plants. The studies were designed to examine patterns of gene silencing [52, 53].

8 Whole Plant FP Applications

8.1 Plant Zygosity Determination Using GFP as a Genetic Marker

One difficulty when working with transgenic plants is transgene zygosity determination of dominant or semi-dominant traits. Using FPs, zygosity status can be determined in two ways. Halfhill et al. [54] demonstrated that heterozygous whole plant GFP fluorescence is approximately half the fluorescence of homozygous plants (Fig. 3a). This finding suggests that zygosity status can be determined by fluorescence alone. In two other studies, tobacco [55] and canola [56] plants expressed GFP under the control of the LAT59 pollen-specific promoter. This promoter was originally isolated from tomato and allows high GFP expression in pollen. The zygosity of these plants was determined based on the ratio of GFP-expressed to non-GFP-expressed pollen. T_1 generation plants were grown and successfully categorized into homozygous (Fig. 3b), heterozygous (Fig. 3c), and isogenic plants for the transgene, according to the relative frequency of GFP-expressed pollen grains [56]. These data

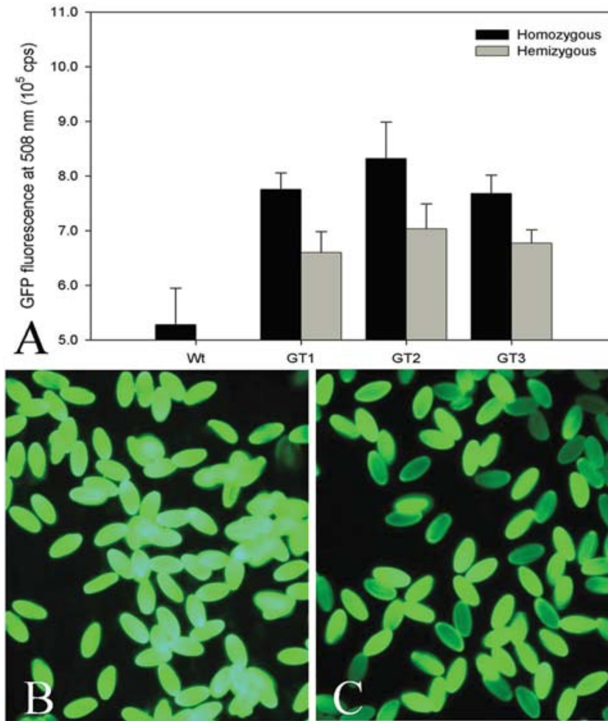

Fig. 3 Trangene zygosity determination. **A** Average GFP fluorescence at 508 nm for wild-type canola (Wt) and three GFP expressing transgenic lines (GT1, GT2, and GT3). Plant material was excited with 385 nm light. **B** Canola pollen from GFP homozygous individual. **C** Canola pollen from GFP heterozygous individual. Zygosity determination is based on the ratio of GFP-expressed pollen. Pictures were taken with blue excitation filter 360/30 nm, 510 long-pass emission filter, 1.54 s exposure time, at 200× magnification

indicate that FPs could be used for ecological monitoring of transgenic plants by analyzing hybridization and introgression status.

8.2 Monitoring Transgenic Organisms

Over the past two decades, transgenic crops have increased in number and acreage grown worldwide. For this reason, concerns over environmental impacts have been voiced [57]. Since many crops are sympatric with their wild relatives, hybridization is sure to occur. It has been documented that transgene escape occurs via seed dispersal or through pollen movement with subsequent hybridization [58, 59]. Therefore, a transgenic monitoring system utilizing FPs to tag whole plants would be useful. In the past, ecological genetics focused on phenotypic traits or DNA markers. However, with FPs one would only have to look for a fluorescent signature. With

this type of monitoring system, FPs can be genetically linked so that they are inherited along with transgenes in the event of gene flow. This would allow the tracking of transgene movement and their ecological effects, an important component in biotechnology risk assessment [60]. This approach has been demonstrated as an effective tool when either whole canola plants [58] or pollen alone [55, 56] were tagged with GFP . When whole plants were tagged, GFP was inherited along with the *Bt* insect resistance gene after transgenic canola was crossed with its wild relative *Brassica rapa* (field mustard) [58]. It has also been observed that GFP and Bt were still present together after several successive back-crosses to *B. rapa* [61]. When pollen was tagged with GFP, it was observed that transgenic pollen captured several meters away could be distinguished from non-transgenic pollen [55, 56]. It would be useful to track transgenic pollen in this manner because the information gathered could then be used in risk assessment. It is also important to note that FP-tagged transgenic plants can be monitored non-destructively, in real time [62], and perhaps remotely [63]. This type of monitoring could be extended to insects, domestic animals, and aquatic organisms.

8.3 Environmental Monitoring

FPs could be used to monitor environmental conditions; in transgenic plants, FPs could be deployed in phytosensors. A phytosenor is a plant that has been genetically engineered to produce a detectable signal, such as an FP, under a certain condition, e.g., to report on a specific contaminant. Since plants are immobile, they possess biochemical and genetic mechanisms to respond to environmental stresses and xenobiotics. Once it has been determined how a particular plant responds to an environmental stress, the information obtained can then be used in the production of a phytosensor. For example, if a gene is expressed at higher levels under a specific condition, the gene's promoter could be cloned and fused to an FP. This construct would then be introduced into a plant to be used as a bioreporter, producing an inducible fluorescence signal. There are many examples of potential phytosensors. In one study, GFP was fused to general stress promoters and introduced into Arabidopsis [64]. Plants were grown under normal conditions and subsequently subjected to low atmospheric pressure. Under low atmospheric pressure, GFP expression was induced contrasting with no GFP under ambient conditions [64]. To alert of herbivory damage, GFP was fused to a peroxidase promoter and introduced into tobacco. After caterpillar species were allowed to feed, GFP fluorescence was detectable [65]. In other studies, pathogen-inducible promoters were fused to GFP [66, 67] and introduced into plants. When these plants were exposed to fungal elicitors, gene expression was reported but at low levels. It is clear that real-world applications of phytosensors are possible; however, none have been deployed on a large scale. There is a real need for biosurveillance in the areas of precision farming systems, integrated pest management, soil fertility, and biosecurity. To make phytosensors a reality, additional research needs to be performed

on plant stress responses and the appropriate FPs need to be determined for optimal expression.

9 Instrumentation and Methods for FP Detection and Quantification in Plants

Observing FPs in whole plants is made possible by their unique fluorescence signatures. These plants can be monitored by simple visual observation or through the use of more sophisticated detection devices. The proper instrumentation is critical when analyzing FPs in transgenic organisms. Here we detail current instrumentation and methods for detection and quantification of FPs in plants.

9.1 Visual Detection

With certain GFP variants, protein expression can be visualized with the naked eye by using a long-wave hand-held UV spot lamp (e.g., UVP model B 100 AP, UVP, Upland, CA, USA) (Fig. 4a). Many GFP variants, such as mGFP5, have dual excitation peaks: one at 395 nm (UV) and another at 470 nm (blue). The human eye cannot see long-wave UV; therefore, we can visualize GFP expression under UV

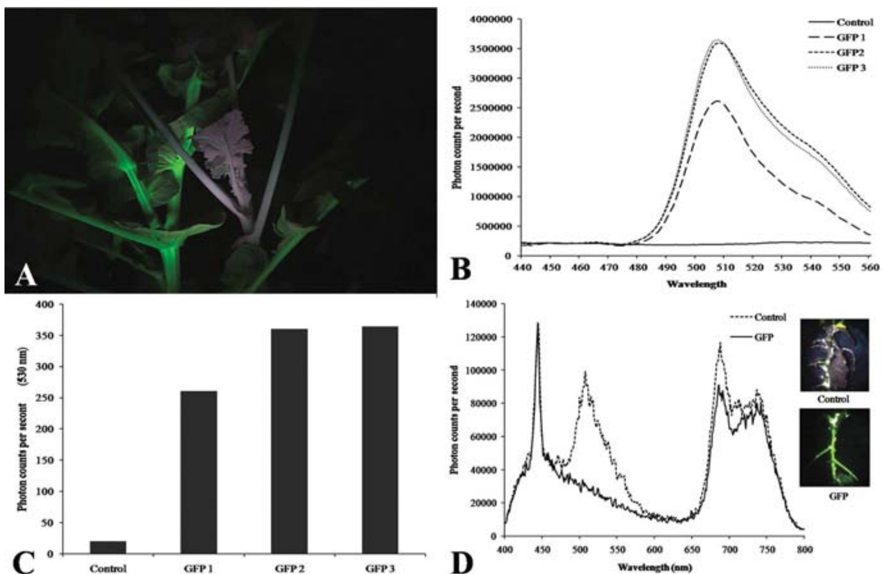

Fig. 4 Examples of current FP detection methods in plants. **A** Visual observation when excited with long-wave UV light (360 nm). **B** Lab-based detection with a FluoroMax scanning spectrometer. **C** Processed reading from the portable GFP-Meter. **D** Stand-off detection with laser-induced fluorescence spectrometry (LIFS) and laser-induced fluorescence imager (LIFI) instrumentation

excitation. Most FPs, including many GFP variants, cannot be visualized in this manner because their excitation maxima are in the visible range. In these cases, visualization requires an emission filter to remove excitation light. An epifluorescent microscope, coupled with the proper filters, is often the best instrument for FP visualization.

9.2 Lab-Based FluoroMax-4 Spectrofluorometer

The FluoroMax-4 (Jobin Yvon and Glen Spectra, Edison, NJ, USA) is a large lab-based scanning spectrofluorometer used to detect and quantify GFP fluorescence. With this instrument, a 2 m bifurcated fiber optic cable is used to transmit excitation light and detect emission transmission to and from the sample [68]. For example, GFP can be measured by exciting a leaf sample with 395 nm UV (UV excitation maxima) light and scanning for emission transmission in the range of 440–600 nm. The collected fluorescence signal is displayed in units of photon counts per second (cps) (Fig. 4b). Standardization and normalization must be performed on each scan because each sample varies in background fluorescence [68]. The FluoroMax can be quantitative as well. In one study, a strong correlation was observed between GFP ELISA quantification and the FluoroMax readings [69]. This detection method will work for all FPs, including the ones with a small Stokes shift. There are excitation filters in place to block out bleed-over light and allow for emission scanning close to the excitation peak.

9.3 Portable Hand-Held GFP-Meter

FP transgenic plants are often grown in field sites far away from the lab. To use a system like the FluoroMax for FP detection, tissue must be collected and brought back to the lab. However, there are field-portable detection systems available such as the GFP-Meter (Opti-Sciences, Tyngsboro, MA) fluorescent spectrophotometer [68]. The GFP-Meter is small, easy to use, operates on a 12 V battery, and has a data logging system. The principle behind the GFP-Meter is similar to the Fluoromax, except it is not a scanning spectrophotometer. Measurements are displayed as a single number in units of photon counts per second (cps) (Fig. 4c). The instrument has a filtered light emitting diode (LED) to generate excitation light. The excitation light travels to a fiber optic cable and through a band-pass filter until it reaches the sample. An attached leaf clip provides stability by holding the sample. The leaf clip also provides consistency between samples by holding the fiber optic cable at a fixed angle. The light emitted from the sample enters back into the fiber optic cable and through a band-pass filter. Subsequently, the measured fluorescence is shown in real time in a display window. The GFP-Meter comes equipped with a 465/35 nm band-pass excitation filter. Two emissions channels are available. Channel 1 is a GFP channel using a 530/35 nm band-pass filter and channel 2 is a chlorophyll channel using

a 680/35 nm band-pass [68]. It is important to note that excitation and emission filters can be changed to meet the requirements of any FP. The GFP-Meter is quantitative as well. Regression analyses on GFP expression between the FluoroMax spectrofluorometer and GFP-Meter measurements produced strong positive correlations [68].

9.4 Stand-Off Laser-Induced Fluorescence Detection

For FP detection from a distance, a laser-induced detection and imaging system has been developed. Laser-induced fluorescence spectrometry (LIFS) and laser-induced fluorescence imager (LIFI) were described and tested with transgenic canola and tobacco expressing GFP [63] (Fig. 4d). LIFS is a laser-based remote detection instrument that records fluorescence of transgenic organisms. The LIFS system collects fluorescence from a 10 cm diameter centered in the laser-illuminated area. A 3-m fiber optic bundle is a conduit for the light collected transferring to the input slit of a 275 cm focal length spectrograph (Model SP-275, Acton Research, Acton, MA, USA). A gated CCD camera (Princeton Instruments, Trenton, NJ, USA) allows detection of the transferred light at the output side of the spectrograph. LIFI is a remote sensing detection system used to capture images of fluorescent organisms. It uses a gated charge coupled device (CCD) camera system (NVSI Camera Systems, Fayetteville, NC, USA) as an imager to collect images. This camera is connected to an intensifier by a fiber optic taper. Fluorescent images of GFP expressing plants can be captured within the intensifier's spectral bands. The bands can be extended from 400 to 900 nm and this emission range would capture the fluorescence of any FP.

These two systems, LIFS and LIFI, have capabilities for remote detection of GFP expression in plants. Any FP could be used with this detection system as long as the excitation laser used meets the requirements of the FP. This capability makes these two systems good candidates for detection monitors of phytosensor systems.

10 Customized FPs

With a variable color palette of FPs characterized, it is no longer novel to clone, mutagenize, and express FPs in transgenic organisms. However, new applications may drive researchers to mutagenize a particular FP to obtain desired spectral characteristics. For example, in one study, mGFP5 was used as a marker for stand-off detection of transgenic plants [63]. A pulsed ND:YAG (neodymium-doped yttrium aluminum garnet) laser with a tripled frequency to 355 nm was used to excite GFP. However, 355 nm has been found to increase the signal to noise ratio about ten times more than the optimal excitation which is 390 nm. Additionally, there is much more endogenous autofluorescence from leaves when excited with lower wavelengths (Fig. 5). When these factors are considered together, GFP fluorescence could be

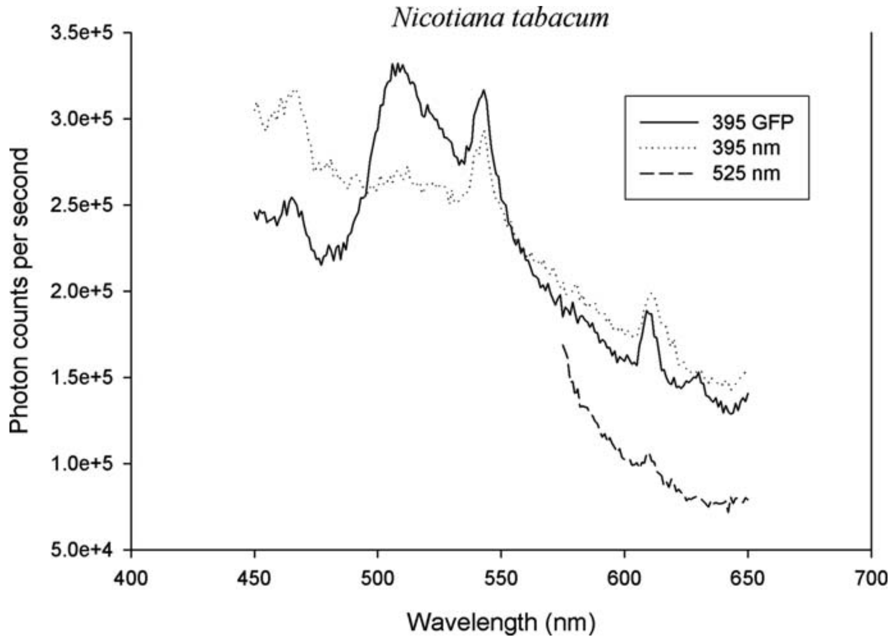

Fig. 5 Background autofluorescence in GFP transgenic and non-transgenic tobacco. Plant leaves were excited by either 395 or 525 nm light. Background fluorescence is much less when exciting with 525 nm compared with 395 nm. These data suggest that an FP with a 525 nm excitation and an emission in the range of 575–600 or 625–650 nm would be ideal in plants

masked and more difficult to detect. To decrease background fluorescence, a doubled frequency ND:YAG laser (532 nm) could be used for excitation. This laser would provide twice the power than excitation at 355 nm and it would significantly reduce the amount of background autofluorescence in plants (Fig. 5). However, this wavelength would not excite GFP. For these reasons, GFP is not the optimal FP for this particular application. If the detection system must be constrained, then it might be most cost-effective to tailor the FP to the laser characteristics. Perhaps an FP exists naturally that meets a priori requirements. Unfortunately, there is no well-characterized native or monomerized FP that meets these requirements (Table 1). Therefore, one will have to be tailored to the application, but laser characteristics are not the only constraints. Plant autofluorescence should be considered as well. There are spectral peaks that are located at 540 nm, which seem to be associated with general plant stress (Stewart et al., unpublished data), 610 and 680 nm, which correspond to chlorophyll fluorescence (Fig. 5). Therefore, a monomerized FP that is excitable at 532 nm and an emission that avoids plant autofluorescence peaks is desirable. There are a number of FPs listed in Table 1 that would be good candidates for random mutagenesis or directed evolution to acquire these characterizations for maximal detection in plant leaves using well-characterized systems.

11 Conclusions

The suite of FPs has dramatically changed scientific research in the past decade and a half. With the many FPs to choose from we can now do things that were not even a thought a few years ago. For example, "brainbow" is an elegant strategy that utilized multiple FPs to map the neural circuits of mice brains [70]. The pictures of the multicolored mice brains are as stunning as the research behind them. With such tools available to researchers, it is certain that the future will be colorful and bright.

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