15

Green Fluorescent Protein Quantification in Whole Plants

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Summary

As future biotechnology applications utilize recombinant proteins as commercial products, nondestructive assays will be necessary to determine protein concentrations accurately within plant tissues. Green fluorescent protein (GFP) has been proposed as a potential marker for the monitoring of transgenic plants and quantifying recombinant protein levels under field conditions. This chapter discusses the utility of using GFP fluorescence as an indicator of protein concentrations and the methods used to quantify GFP fluorescence in whole plant tissues. Furthermore, we discuss the accuracy and effectiveness of the portable General Fluorescence Plant Meter (GFP Meter, Opti-Sciences, Inc.) compared to a laboratory-based spectrofluorometer (Fluoro-Max2, Jobin Yvon & Glen Spectra). In whole plants, GFP fluorescence was shown to be variable at each leaf position over time and among different leaves on the same plant. A leaf had its highest GFP fluorescence after emergence, and subsequently, its fluorescence intensity decreased over time. Younger leaves were significantly more fluorescent than older leaves on the same plant. GFP fluorescence intensity was directly correlated with the concentration of soluble protein per unit wet mass and with another genetically linked recombinant protein (*Bacillus thuringiensis* [Bt] *cry1Ac* endotoxin protein).

Key Words: *Bacillus thuringiensis* (Bt); green fluorescent protein (GFP); soluble protein concentration; spectrofluorometer; transgene monitoring.

1. Introduction

Monitoring transgenic plants under field conditions will become increasingly important as various new genetically modified (GM) crops are implemented in large-scale agriculture. At present, recombinant proteins produced within GM crops provide important production characteristics to plant cultivars, such as

From: *Methods in Molecular Biology, vol. 286: Transgenic Plants: Methods and Protocols*
Edited by: L. Peña © Humana Press Inc., Totowa, NJ

herbicide tolerance and insect resistance, but the proteins themselves are not commercial products. In many future applications, however, recombinant proteins produced within plant materials will be economically important products. Future transgenic plants will function as "biofactories," and will manufacture a wide array of products ranging from pharmaceuticals to plastics. Real-time, nondestructive assays to determine the concentrations of these economically important recombinant proteins will be essential technologies for the profitable use of future biotechnology products.

For a monitoring system to be effective, the genetic marker technology should be accurate with few false positives and negatives, detectable throughout the life cycle of the plant, and able to inform on the status of genetically linked or fused transgenes of interest. Green fluorescent protein (GFP) has been proposed as a whole-plant marker for field-level applications *(1)*. The GFP gene was first cloned from jellyfish (*Aequorea victoria*) in 1992, and has since been modified for specific applications and transformed into many different organisms *(2–5)*. GFP monitoring has the potential to track transgenes under large spatial scales utilizing visual or instrumental detection of the characteristic green fluorescence of transgenic material. The *mgfp5-er* variant gene has been shown to be a feasible transgene monitor in plants under field conditions *(6,7)*. This gene was field-tested in tobacco (*Nicotiana tabacum*), and the plants synthesized the protein and remained fluorescent throughout the growing season *(7)*. GFP has also been shown to be a feasible qualitative marker for the presence of a linked synthetic *Bacillus thuringiensis* (Bt) *cry1Ac* endotoxin transgene *(7,8)*. With these beneficial characteristics, the next step in the development of a GFP monitoring system is to better describe the system and resolve weaknesses that could limit the utility of the monitoring system.

From our current research, GFP fluorescence in GFP transgenic plants followed two specific patterns *(9)*. First, fluorescence intensity of 530-nm green light decreased at each leaf position over time. Second, fluorescence differed among leaf positions on the same plant with the highest fluorescence observed in young leaves. GFP fluorescence intensity was highest in young leaves up to 2 wk after emergence, then the fluorescence intensity decreased over time to levels observed in nontransgenic controls as leaves aged. Therefore, when a plant has a large number of leaves at various ages, a wide spectrum of GFP fluorescence can be detected, ranging from the highest level observed in that transgenic event at and near the apical meristem to those similar to nontransgenic levels in old leaves. In leaf tissues, in which the cauliflower mosaic virus 35S promoter produces a consistent percentage of recombinant protein per unit total soluble protein *(10,11)*, GFP fluorescence consistently varied with the concentration of soluble protein in a mass of fresh leaf tissue. This research

has shown that soluble protein per unit leaf mass changes during leaf development, and the GFP phenotype is correlated with this phenomenon.

One of the key aspects of using GFP fluorescence in a monitoring system for transgene expression is that it accurately quantifies the concentration of genetically linked proteins of interest. The results from current experiments showed that GFP fluorescence accurately predicted the concentration of Bt, even as both parameters changed over time *(9)*. However, the results also imply that false negatives could be generated if one solely assays older leaves having lower concentrations of soluble proteins. That problem can be minimized if GFP fluorescence measurements are made on younger leaves near the apical meristem. In addition, it has been known for some time that sequential senescence and the decline in soluble protein proceeds at a faster pace under water stress or nitrogen deficiency *(12)*. Therefore, the observation that changes in GFP fluorescence were correlated with those in soluble protein concentration may prove to be a significant tool for monitoring particular plant stresses.

There are several types of systems presently in use for the macroscopic detection and quantification of fluorescent compounds including: high-intensity UV lamps, spectrofluorometers (e.g., FluoroMax-2, Jobin Yvon & Glen Spectra, Edison, NJ), and scanning laser systems (e.g., FluorImager, FluorImager SI, Molecular Dynamics, Sunnyvale, CA). A handheld 365-nm UV lamp, such as a UVP Model B 100 AP (UVP, Upland, CA), allows for expeditious scanning of GFP fluorescence in whole plants. However, the UV light must be used in darkness, it is only effective for UV excitable GFP variants, and visual observation cannot be used to quantify GFP fluorescence. Spectrofluorometers and fluorescence imaging systems are capable of detecting the presence of GFP and also allow for quantification of fluorescent tissues *(7,8,13–15)*. However, both systems are laboratory-based and expensive. For several years, plant researchers have sought a portable instrument that measures GFP in field plants under ambient lighting conditions. Millwood et al. *(16)* described the methods used to quantify GFP fluorescence with the Opti-Sciences General Fluorescence Plant Meter (GFP Meter). Performance of the GFP Meter was compared to the Fluoromax-2, a validated GFP fluorescence measurement tool *(7,8,13)*. A comparison of instantaneous measurements from the GFP Meter (530-nm wavelength) and the Fluoromax-2 (standardized 510-nm wavelength fluorescence values) was completed to gage accuracy. The regression analyses of growth chamber-grown GFP canola, greenhouse GFP tobacco, and field-grown GFP tobacco produced high R^2 values (0.87, 0.88, and 0.89) and indicated a positive functional relationship between instruments.

This chapter discusses the methodology used to quantify GFP fluorescence in whole plant tissues. The two GFP detection systems that we use are introduced, and we describe how to collect and analyze the in vivo plant fluorescence values

produced from these spectrofluorometers. We also introduce the method used to correlate GFP fluorescence and another genetically linked recombinant protein of interest (Bt). In the **Notes** subheading, we discuss problems that might arise with measuring GFP fluorescence and how plant health and life cycle status play an important role in GFP fluorescence.

2. Materials

2.1. GFP Meter

The GFP Meter (Opti-Sciences, Tyngsboro, MA) is a self-contained, field-portable fluorescence detection and data logging instrument powered by an internal 1.2-ampere h 12-V gel lead acid battery (**Fig. 1**). A filtered light emitting diode (LED) generates excitation light-when powered on. A small portion of this light is monitored to compensate for temperature drift. The output of the LED is focused on one of three fiber ports. A driver, controlled by a microprocessing unit (MPU), regulates the LED power level and compensates for changes in battery voltage. This excitation light travels through a bandpass filter to a fiber-optic cable and is then delivered to the sample. Attached to the end of this cable is a leaf clip, which has been installed to keep the cable in place. The light emitted from the sample enters back into the fiber-optic cable and is directed through a bandpass filter into a low-noise preamplifier. This signal is then fed into an analog/digital signal processing and filtering unit slaved to the MPU. Fluorescence measurements appear in real time on a liquid crystal display in units of counts per second (cps). A 12-selection keypad provides user management of test functions and setup. A nonvolatile memory chip (capable of storing 1020 sample points) assures that data will not be lost when power is turned off or the battery removed and a RS-232 port enables downloading data to a computer. The GFP Meter uses a modulated detection system to minimize the effects of temperature drift and stray light. Virtually any bandpass filter combination can be used for excitation and emission. For this study, a 465-nm filter with a bandwidth of 35 nm was used for excitation. Channel 1 (GFP channel) used a 530-nm filter with a bandwidth of 35 nm for emission and channel 2 (chlorophyll channel) used a 680-nm filter with a bandwidth of 35 nm for emission. Chlorophyll data are not reported in this study.

2.2. FluoroMax-2

The FluoroMax-2 (Jobin Yvon & Glen Spectra) is a laboratory-based spectrofluorometer system that uses a computer to process data. All FluoroMax-2 functions are under control of Datamax spectroscopy software (Galactic Industries Corporation, Salem, NH). Light from a 150-W xenon lamp enters an excitation spectrometer, which delivers monochromatic light to a bifurcated fiber-optic cable. A rubber protector surrounds the external end of the cable and extends 4

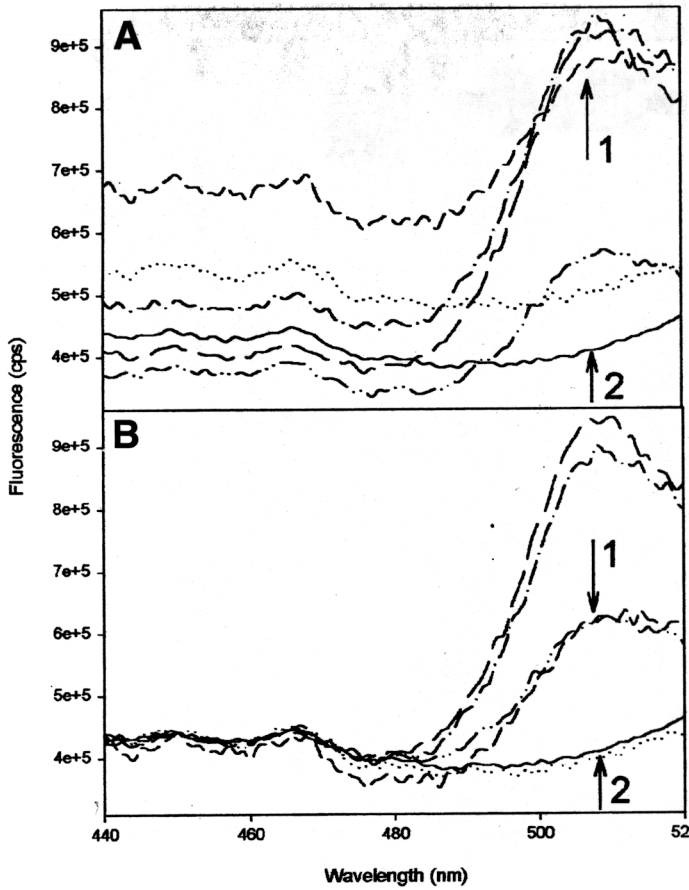

Fig. 1. Standardization of spectrofluorometer (FluoroMax-2) measurements of field-grown GFP tobacco excited with 385-nm UV light. Each line represents an individual tobacco plant. When looking at the GFP maxima (508 nm) of nonstandardized spectral data (**A**), three plants appeared to be expressing high amounts of GFP with respect to the other plants analyzed. However, after standardization (**B**) to a nontransgenic control *(2)*, it is revealed that one of these plants *(1)* is actually expressing lower levels of GFP.

mm beyond to prevent signal disruption from dirt and debris and to provide a dark environment for measurements. Light flows through the cable only when a sample is being scanned. When scanning, the cable is then placed onto the sample and light emitted from the sample flows back through the fiber-optic cable to the emission spectrometer where it is dispersed and directed to a signal photomultiplier detector. This fluorescence signal is then amplified and displayed on a computer monitor in units of cps.

2.3. GFP Plant Material

In our research, plants transgenic for GFP (*mgfp5-er*) (*6*) and GFP/Bt (*mgfp5-er/Bacillus thuringiensis*) under the control of constitutive *CaMV 35S* promoter were used (*7–9*). This GFP variant is excited equally by both blue (465-nm) and UV (395-nm) wavelengths. We have experience with three species of GFP transgenic plants: canola (*Brassica napus* cv. Wester), tobacco (*Nicotiana tabacum* cv. Xanthi), and *Arabidopsis thaliana* ecotype Columbia. Nontransgenic control plants from the original plant cultivar or ecotype were also included in each experiment. The zygosity status (homozygous, hemizygous, or mixed) of each line was determined in previous research (*see* **Note 1**).

2.4. Bt Enzyme-Linked Immunosorbent Assay (ELISA)

The EnviroLogix Cry1Ab/Cry1Ac Plate Kit is designed for the quantitative laboratory detection of Cry1Ab and Cry1Ac residues in corn and cotton leaf tissue samples (Cry1Ab/Cry1Ac Plate Kit, cat. no. AP 003, Envirologix, Portland, ME). Previous research within our laboratory has also shown this kit to be accurate for the determination of Bt concentrations from canola leaf tissue samples.

2.5. Protein Extraction from Leaf Tissues and Soluble Protein Quantification

0.1 *N* NaOH and 1 *M* Tris-HCl, pH 4.5, were used as extraction buffers. Bradford analysis was performed using Bio-Rad Protein Assay Dye Reagent Concentrate (cat. no. 500-0006, Bio-Rad Laboratories, Hercules, CA).

3. Methods

3.1. GFP Fluorescence Quantification

1. Plant lines were germinated and grown under the same environmental conditions. The plant conditions varied based on the experimental design. Optimal plant health was important in GFP experiments (*see* **Note 2**), and therefore, conditions were selected based on the experimental plant species (*see* **Notes 3** and **4**).
2. After a specified time period, the plants were selected for GFP analysis. It was important to evaluate plants that were at the same developmental stage (*see* **Note 5**). The same leaf position was chosen for each individual plant. Time course studies were also an option, and in this case, the same leaf positions were tested at consistent time intervals (between 1 and 7 d) over the course of the experiment.
3. A spot (1-cm diameter, the size of the end of the fiber-optic cable) on the underside of sample leaves and adjacent to the leaf mid-vein was excited at 465 nm with the GFP Meter or 385 nm with the Fluoromax-2. Two fluorescence mea-

surements were recorded on opposite sides of the mid-vein on the underside of each leaf.
4. The GFP Meter recorded single 530-nm GFP fluorescence and 680-nm chlorophyll values. The Fluoromax-2 recorded a selectable emission range, and in our experiments included 420–600 nm.
5. Emissions spectra were recorded from five individual control plants and were averaged. The average control was used to standardize the Fluoromax-2 data.

3.2. Fluorescence Standardization

1. The FluoroMax-2 GFP fluorescence scans were standardized to control samples to account for baseline variation of each leaf measurement (*see* **Note 6**). The protocol for standardization involves selection of a wavelength outside the GFP fluorescence spectrum as a point of normalization for each FluoroMax-2 scan. For this study, the 450-nm wavelength was the anchor point.
2. Subsequently, each emissions scan was standardized to the average control for that species. Functionally, the 450-nm GFP value of the sample was subtracted from the 450-nm average control value. The resulting integer was then added to each wavelength value along the sample spectra (420–600 nm). This method eliminates differences outside the GFP emissions spectra, allowing for comparison of GFP magnitudes.
3. The GFP Meter did not have the option of standardization because the appropriate filter sets outside the GFP emissions range were not installed for this study.
4. The standardized GFP fluorescence values were used to compare treatments within each experiment.
5. In our experiments, GFP value for each leaf position was the unit of measure compared between treatment types. For example, the average GFP value at the fourth leaf position between plants could be compared for each experimental treatment.

3.3. GFP Fluorescence Correlations With Several Protein Concentrations

1. GFP fluorescence measurements were recorded as described in **Subheading 3.1., step 3.**
2. For the transgenic samples, two 1-cm diameter leaf punches were collected from fresh leaves at the same position as the GFP measurements from each leaf with a 1.5-mL microcentrifuge tube.
3. The mass of each punch was recorded, and was then flash frozen in liquid nitrogen (LN). The sample was homogenized with 0.5-mm glass beads in a mechanical amalgamator (Silamat S5, Ivoclar Vivadent Clinical, Austria).
4. After disruption of the leaf tissue, 400 mL of 0.1 *N* NaOH was added to each sample, and the sample was incubated on ice for 30 min. After incubation, 80 mL of 1 *M* Tris-HCl, pH 4.5 was added to each sample for neutralization. The sample was centrifuged for 7 min at 6200*g* (Denville 260D Microcentrifuge, 24 sample rotor, Denville Scientific, Metuchen, NJ), and the supernatant containing soluble protein was recovered.

5. Bradford Analysis (Bio-Rad Laboratories) was used to quantify soluble protein concentration in 96-well plates (EL 800 Universal Microplate Reader with the KC Junior software package, Bio-Tek Intruments, Winooski, VT).
6. Bt protein concentration was determined by the use of a Bt ELISA.
7. Regression analysis was performed for standardized GFP fluorescence per leaf sample by the concentration of soluble protein and Bt per unit wet mass (StatView 5.0 for Windows). The protein concentrations determined by these methods may also be compared with standard curves produced for each specific protein (*see* **Note 7**).

4. Notes

1. The zygosity status (either homozygous or hemizygous), for the experimental plants must be understood prior to the start of the experiment. In previous research, the GFP gene demonstrated additive transgene expression in 10 independent transformation events of canola *(9)*. In all canola lines, homozygous individuals that contained two copies of the transgene locus fluoresced twice as much as hemizygous individuals above the background level of fluorescence. We caution against using segregating populations for any fluorescence-based experiments, because the plants may exhibit a wide range of fluorescence based on the number of transgene copies. Experimental treatments will rarely play a larger role than the zygosity status of the plant, and we suggest using strictly homozygous populations if GFP fluorescence is going to be measured between treatments.
2. Plant health is vitally important to utilize GFP fluorescence as a quantifiable tool. Sick or stressed plants will not have robust and reliable GFP fluorescence. Independent GFP plant lines may exhibit a wide range of fluorescence based on the plant health, and the introduction of any unintended plant stress during the course of the experiment will likely reduce the repeatability of the results. Plant stress caused by nutrient, water, or light deficiencies will dramatically alter the concentration of soluble protein in the leaf tissues. Several experiments *(9–11)* indicate the percentage of GFP within extractable soluble protein is expected to remain consistent, and therefore, the changes in GFP fluorescence in plant tissues is caused by changes in soluble protein concentration. If the plants undergo a period of stress and modulate soluble protein production, the GFP measurements will reflect this change and potentially invalidate the experiment.
3. The environmental conditions for all plants of the experiment must be highly consistent to compare the data between treatments. Variations between different environmental chambers, greenhouse rooms, or greenhouse locations can cause significant differences in GFP fluorescence, once again invalidating the data. Many experiments using large numbers of plants by necessity must use more than one location, and growing plants in different locations have caused significant location based in error in some of our preliminary work. Randomization of experimental treatments does not solve location-based variance, because the location effect often can be the largest cause of error in GFP fluorescence-based experiments. We suggest that emphasis on environmental and experimental standardiza-

tions during the development of the experimental design will lead to repeatable results.

4. Overall, the utility of GFP fluorescence as an analytical tool is limited to relative comparisons between plants of the same species grown under similar conditions. We have found that it is difficult to compare the actual GFP values between species and cases where the plants are grown in disparate conditions, that is field and in the laboratory. With this in mind, the experiments must have the appropriate controls to estimate relative changes in GFP fluorescence between treatments, and then the trends that are detected may be used to understand the differences in fluorescence between plant species and environmental conditions.
5. The plant life history stage alters the degree of GFP fluorescence for each plant tissue, and selecting tissues from the same stage is important in GFP experiments. The location of detectable fluorescence changes dramatically as a plant progresses through its life cycle. The 35S promoter produces high expression of GFP in young leaves and shoot meristems, and GFP yields similar patterns as GUS under the control of identical promoters *(11)*. When plants have a large number of leaves, a full range of GFP fluorescence can be seen with the brightest possible young leaves to older leaves with wild-type levels of fluorescence. In mature plants, green fluorescence was detectable in meristems. GFP fluorescence has been shown to be visible in young leaves, stems, veins, and flowers, and specifically selecting tissues at the same stage of development will allow comparisons between treatments.
6. The spectrofluorometers used in this study may add variation to the GFP fluorescence measurements based on the techniques used by the experimenter. Both instruments use a cable to deliver the excitation light to the leaf surface, and different experimenters may add significant variation to the data by their interpretation of the methods. In our case using the Fluoromax-2, we have seen that the angle the cable is held in relation to the leaf surface can affect the magnitude of the GFP value. We have found that it may be beneficial to standardize the angle by clamping the cable in a fixed orientation. This potentially solves this problem and allows multiple users to produce similar GFP values. For the GFP Meter, the amount of time the cable is clipped to the leaf prior to excitation has been shown to change the GFP magnitude. We suggest that the GFP value should be measured rapidly after the leaf clip is placed on the leaf.
7. Producing standard curves for known amounts of GFP may be useful, because this procedure allows for in vivo estimations of protein amount based on GFP fluorescence values. Richards et al. *(17)* reported that the fluorescence intensity increases linearly as the amount of GFP increases. The resulting standard curves were then used to estimate the amount of GFP in unknown samples, in this case either protein extracts or direct leaf measurements. Data from ELISA supported the validity of the fluorescence-based estimates. In fact, it was possible to generate recombinant protein estimates *in planta* because the fluorescence properties of the intact leaf did not affect the GFP signal. This technique may facilitate future characterization of GFP and GFP-fusion transgenic plants by eliminating the need for laboratory-based protein quantification methods.

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