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Patterns of Green Fluorescent Protein Expression in Transgenic Plants

BRIAN K. HARPER[1] and C. NEAL STEWART JR.[2],*

[1]*Novartis Agricultural Biotechnology Research, Inc., 3054 Cornwallis Rd., Research Triangle Park, NC 27709;* [2]*Dept of Biology, University of North Carolina, Greensboro, NC 27402-6174*

Abstract. Modified forms of genes encoding green fluorescent protein (GFP) can be macroscopically detected when expressed in whole plants. This technology has opened up new uses for GFP such as monitoring transgene presence and expression in the environment once it is linked or fused to a gene of interest. When whole-plant or whole-organ GFP visualization is required, GFP should be predictably expressed and reliably fluorescent. In this study the whole plant expression and fluorescence patterns of a mGFP5er gene driven by the cauliflower mosaic virus 35S promoter was studied in intact GFP-expressing transgenic tobacco (*Nicotiana tabacum* cv. Xanthi). It was shown that GFP synthesis levels in single plant organs were similar to GUS activity levels from published data when driven by the same promoter. Under the control of the 35S promoter, high expression of GFP can be used to visualize stems, young leaves, flowers, and organs where the 35S promoter is most active. Modified forms of GFP could replace GUS as the visual marker gene of choice.

Key words: expression patterns, green fluorescent protein, marker genes

Introduction

Since the discovery of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* it has become a frequently used tool in biology. GFP is a 27 kDa protein that fluoresces green when excited by ultraviolet or blue light (Heim and Tsien, 1996). GFP does not require the addition of a substrate or cofactors for fluorescence to be detected and it has been expressed in a number of organisms. Until recently, however, heterologous expression in plants had been minimal due to the splicing of a plant recognized cryptic intron (Haseloff et al., 1997). Modifications have since been made to the plant recognized splice sites in order to achieve proper GFP expression in plants.

One of these modified versions of GFP that is properly expressed in plants is the mGFP5er variant that, along with modified splice sites, is also targeted to the endoplasmic reticulum by the addition of a N terminal arabidopsis basic chitinase fusion and a C terminal HDEL fusion (Haseloff et al., 1997). The coding sequence contains three mutations giving the GFP novel properties. The V163A and S175G mutations enhance the folding of mGFP5er at higher temperatures

*Author for correspondence. e-mail: nstewart@uncg.edu; ph: 336 334 4980.

while an I167T mutation gives the ultraviolet (395 nm) and blue light (473 nm) excitation maxima equal amplitudes (Siemering et al., 1996). With advancements made in GFP coding sequence modifications such as that in mGFP5er, GFP is now macroscopically detectable at the whole plant scale (Stewart 1996; Leffel et al., 1997; Harper et al., 1999). Previously, GFP has mainly been used in studies concerning gene expression at the cellular level, protein trafficking, and protein-protein interactions (Chalfie et al., 1994; Grebenok et al., 1997; Mahajan et al., 1998). Now, macroscopic detection allows novel uses for GFP at the ecological level.

We believe that GFP will be an important tool for real-time monitoring of plants grown in the field. One prerequisite for this technology is that, once GFP is driven by a promoter of choice, its expression will be both reliable and predictable. Also, by characterizing whole plant expression patterns, it would make results more applicable while monitoring GFP transgenics in the field. For example, when using a portable fluorescence spectrophotometer, it is desirable to take one measurement per plant to assay for trangenicity. Thus, it is important to find a leaf or other part of the plant that produces the optimal and most meaningful reading.

The cauliflower mosaic virus (CaMV) 35S promoter is a strong constitutive promoter that is commonly used in the expression of transgenes in plants (Hemenway et al., 1988), and was used to drive the mGFP5er gene in transgenic plants for this study. Both the functional structure of the CaMV 35S promoter and many of its activity level patterns in plants have been well characterized (Benfey et al., 1989; Odell et al., 1995).

The objective of this study was to compare mGFP5er expression patterns to learn whole plant expression patterns of GUS (beta-glucuronidase) driven by the CaMV 35S promoter. In this study, field-grown transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi) expressing mGFP5er (driven by the CaMV 35S promoter) were characterized for GFP synthesis and fluorescence levels in different plant organs of whole plants.

Materials and Methods

Experimental plants

Three mGFP5er transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) plants (two grown in growth chambers and one grown in the field) and two control (non-transgenic) plants were used for whole plant expression analysis. The plants were grown in the field for approximately 4 months. Plants grown under artificial lights were approximately 8 weeks old when data was taken and were grown under fluorescent lights in a 18 h photoperiod at 22ºC until they were 6 weeks old. At this point they were moved under high-output halogen lights (12 h photoperiod) for 2 weeks. The plants were allowed to mature so that all organs of various ages could be sampled.

Fluorescence spectrometry

Fluorescence spectrometry was performed on various organs from different regions of plants, including leaves from the top through the lower leaves and their

midveins. Fluorescence measurements were taken on upper, middle, and lower regions of the main shoots. Not all measurements (including flower and mid-vein measurements) were made on some plants. Spectrometry readings were taken from the center portion of the leaf (off and left of the midvein). Spectrophotometry readings were done using a Fluoromax-2 fluorescence spectrophotometer from Instruments S.A., Inc.; Edison, NJ using DataMax and GRAMS / 386 software (by Galactic Industries Corporation Salem, NH). This spectrophotometer uses a 2 m fiber optic cable so that fluorescence measurement can be taken directly on the plant. Transgenic plants containing the mGFP5er gene and non-transgenics were excited at 385 nm and emission was detected at 509 nm.

Protein analysis

Western blot analysis was performed on the same tissue used for fluorescent spectrometry. After fluorescence was measured, approximately 0.15 g of tissue per sample was used for western blot protein extraction. Samples were taken from the center (excluding midveins) of leaves in which multiple or single spectrometry readings were made. Tissue samples were homogenized in 500 μL of extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol, 10 mM EDTA pH 8.0, 18% glycerol) using a plastic pestle driven by a homogenizer (approximately 800 rpm). Extracts were centrifuged at 16,000 *g* for 10 min and the supernatant was taken to remove excess plant material. Total protein was quantified using the Bradford protein assay. 20 μg of total protein was used in each sample well combined with 1:1 ratio of Laemmli buffer (Laemmli, 1970) and separated on 5% (stacking) and 10% (separating) polyacrylamide gels. Protein transfer to nitrocellulose membrane was carried out in a wet tank (containing tris base, glycine, methanol, and water) for 4 h at 35 v. Immunostaining was carrried out by using 1:3000 anti-GFP serum developed in rabbit (Clontech, Palo Alto, CA), 1:1000 secondary goat anti-rabbit (Sigma, St. Louis, MO), and 1:1000 rabbit anti-goat alkaline phophatase conjugated complex (Sigma). GFP was detected on blots by exposure to nitroblue tetrazolium/bromochloroindolyl phosphate. Recombinant GFP (Clontech) amounts (0.2, 2, and 20 ng) were used as protein standards. Western blots were scanned (Hewlett Packard ScanJet 4°C) and intensities of GFP bands were compared with standards to determine GFP amounts in each sample. The percent of total protein represented by GFP was then calculated [% GFP = (ng GFP in sample / 20,000 ng total protein added) x 100].

Chlorophyll assay

Chlorophyll content was measured in 4 leaves of each plant from top to bottom for one artificial light grown mGFP5er transgenic plant and one non-transgenic plant. Chlorophyll was estimated using a Minolta SPAD-502 Chlorophyll Meter (Spectrum Technologies Inc., Plainfield, IL) which determines relative chlorophyll content in plant leaves. The instrument calculates a ratio based on chlorophyll's' relative absorbance of red (640 nm peak wavelength) and infrared (940 nm peak wavelength) light passing through a leaf sample.

Figure 1. Wild-type (red, left) and mGFP5er transgenic (green, right) tobacco plants grown under artificial light. Both plants are excited under ultraviolet light (365 nm) using a hand held UV light (UVP model B-100AP 100W). Picture was taken with 100 ASA film at f-stop 11 and a 60 s exposure with a yellow filtered macro lens.

Results

GFP patterns on plants grown under artificial lights

One mGFP5er transgenic (plant line A) was extensively characterized when 5 of its leaves (including mid veins) on both sides from top to bottom were analyzed. Roots, 3 shoot locations (high, medium and low), calyx, and leaf primordia were also analyzed (Figures 1 and 2). Similar sampling was performed on a non-transgenic plant. Trends showed that both fluorescence and GFP synthesis levels were greatest in vascular material and younger leaves (Table 1, Figure 3). Also, all shoot and root GFP concentrations were slightly higher than those found in leaves, but similar to vein GFP concentrations. Regression analysis between multiple % GFP concentrations and fluorescence spectrometry readings from non-transgenic tobacco plants grown in artificial light and the preceding mGFP5er transgenic showed a strong linear relationship (P=0.0004 R^2=0.6357).

Data from a mGFP5er transgenic of a different transgenic line (B) (protein blots: three leaves, roots, flowers and spectrophotometry: 3 shoot locations, flowers) also indicate that GFP expression may be slightly higher in vascular (shoots in this case) and in younger tissue. Chlorophyll content (in relative units) of leaves from top to bottom in one mGFP5er transgenic plant sampled was 33.8, 38.1, 28.0, 34.1 and 46.1 respectively as compared to 31.7, 30.2, 28.8 and 36.0, of a sampled non-transgenic plant. Percent GFP of total protein for three leaves from top from bottom were 0.15%, 0.12% and 0.12% respectively. Shoot spectrophotometry readings from top to bottom were 2,300, 1720, and 1,300 cps respectively. Both flower (0.175%) and root (0.2%) GFP levels were higher than leaf GFP synthesis levels.
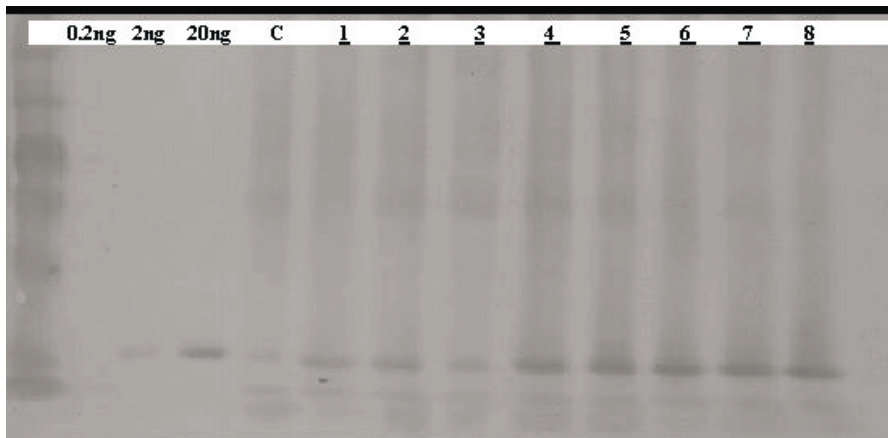
Figure 2. Whole plant western blot analysis of artificial light grown mGFP5er transgenic plant. Represented are 0.2, 2, and 20 ng GFP standards, a control leaf sample and blots from various plant organs including leaves (rows 1-3), capsule (4), high, medium and low shoot locations (5,6,7) and leaf primordia (8).

Table 1. Comparison of GFP synthesis and fluorescence in a GFP transgenic plant grown under artificial light. Values are for high and low GFP-expressing tissues and specific measurements (GFP % of total protein or fluorescence) as well as p values at 0.05 significance.

P	High	Low	Measurement
0.0437	Vascular	Leaf	Fluorescence
0.0008	Vascular	Leaf	%GFP
0.0002	Midvein	Leaf	%GFP
0.0002	Higher Leaves	Lower Leaves	%GFP

Field grown plant expression patterns

A field-grown plant of the same mGFP5er transgenic line was also similarly characterized (Figure 4). Expression levels this time were found to be the same (P=0.4747) for vascular tissue (shoots and roots) and leaves. Fluorescence spectrophotometry measurements taken on the middle and top (adaxial surface) of leaves between the field grown non-transgenic and the mGFP5er transgenic were not significantly different (P=0.217). Multiple single leaf fluorescence spectrophotometry showed that fluorescence levels were significantly higher on the bottom of the leaf than on the top (P=0.001). Measurements on each side of the non-transgenic plant leaves also showed that spectrophotometry readings were slightly higher although not significantly so (P=0.1168). Spectrophotometry data from three leaves of the field grown GFP transgenic top to bottom indicated that the readings on the adaxial surface of leaves closer to the main shoot were slightly higher (only 107 cps) than those taken further away from the shoot.
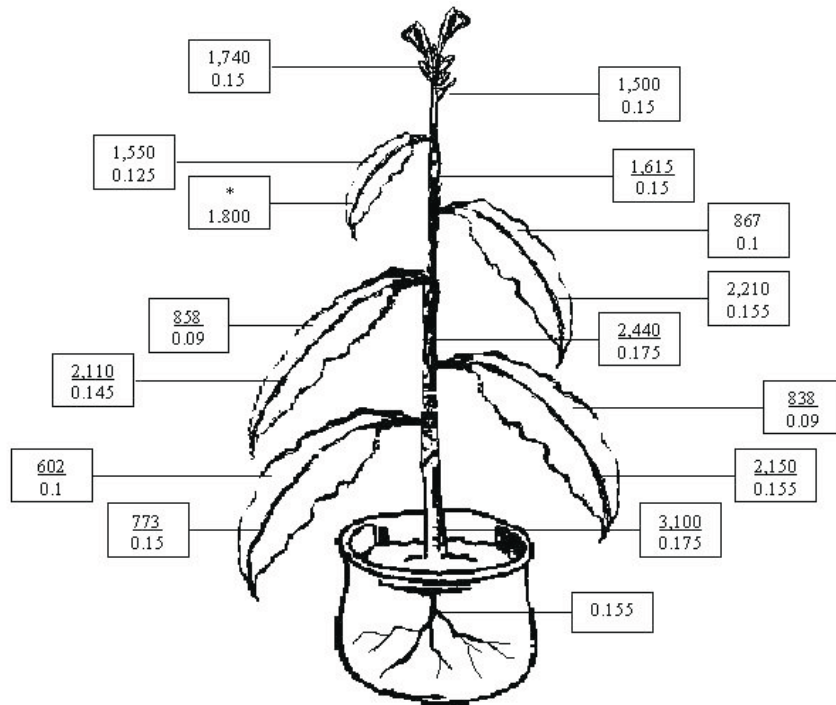
Figure 3. GFP synthesis and whole plant fluorescence patterns of a lab grown mGFP5er transgenic plant (line A). Underlined (top values) are fluorescence spectrophotometry readings (counts per second x 1,000) bottom values indicate percent GFP of total extractable protein as found by western blot analysis. Values within leaves represent mid vein measurements, values outside of leaves represent measurements taken on the leaf itself (top, center). Capsule and leaf primordia measurements are also included. * represents missing values.

Discussion

It is important to be able to predict the pattern of GFP fluorescence in different plant organs of various ages if GFP is to be used for agricultural and ecological applications. This predictability could be useful for constitutive promoters, to monitor transgene presence, and for inducible promoters, with applications such as environmental monitoring. It is also important to show that GFP has no characteristics that would otherwise prevent its synthesis in certain plant organs. This would be of concern, for example, for transgenic crops in which GFP was to be targeted away from certain organs or tissues.

We used few plants for this study because we were interested in spatial and tissue specific patterns of GFP expression and fluorescence. In a related study (Harper et al., 1999) hundreds of protein and fluorescence measurements were made on individual plants from the same transgenic line used in this field study. In the field study where the third leaf to plant apex was sampled, percent GFP of total plant protein levels (0.186 to 0.028% GFP of total protein) were similar to what was seen with the mGFP5er transgenic the plant of the same line grown in the field for this study (Figure 3). Percent GFP of total protein at midseason
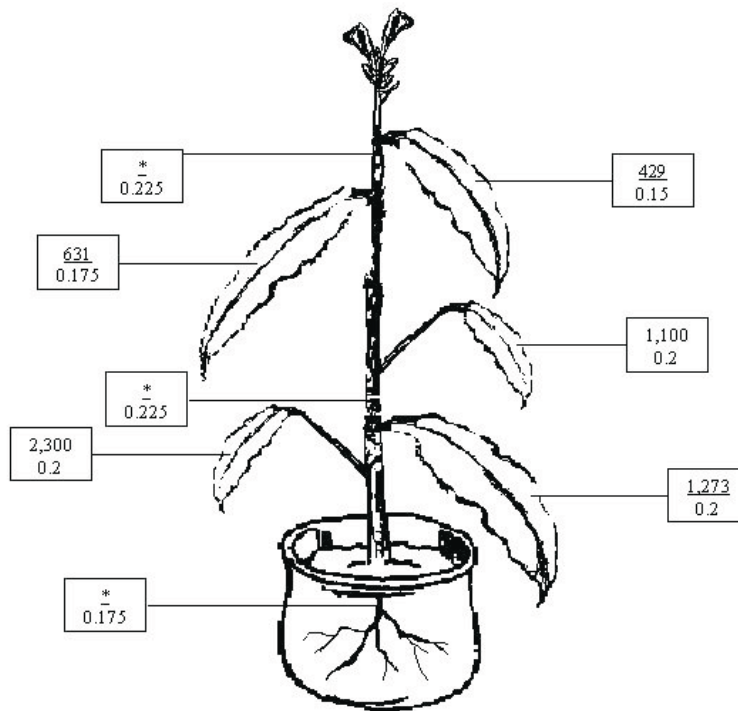
Figure 4. GFP expression and whole plant fluorescence patterns of a field grown mGFP5er transgenic plant (plant line A). Underlined (top values) are spectrophotometry readings (counts per second x 1,000) bottom values indicate percent GFP of total extractable protein as found by western blot analysis. Measurements represented are those taken on 2 shoot locations, roots, 3 separate leaves from the main shoot and leaves from separate axillary shoots. * represents missing values.

(0.109 to 0.47%) from plants of the same line grown in the field study were also similar to the percent of GFP from the younger plants under artificial light (Figure 3). Given that GFP synthesis amounts were similar within the same line for the two studies, and that mean GFP synthesis amounts of that line did not show a significant difference from other the two other highly fluorescing lines (0.172 to 0.047% and 0.111 to 0.059% GFP of total extractable protein) (Harper et al., 1999) in the field, it may be assumed that the expression patterns seen here would be comparable with other GFP transgenic lines in other situations.

Our data indicated that GFP expression was detectable from all plant parts that were tested, including leaves, leaf mid-veins, shoots, roots and flowers from plants grown both in the lab and the field. This would be expected as the CaMV promoter has been shown to be active in most plant organs (Benfy et al., 1989), and during most stages of plant development (Odell et al., 1985). In the artificial light grown mGFP5er transgenic plant it was shown that GFP content was higher in vascular tissue than in leaf material. Similar expression patterns have been described when using the CaMV 35S promoter in tobacco (*Nicotiana tabacum*) using GUS as a marker gene. Using this system, Jefferson et al. (1987) found higher levels of GUS activity in roots and stems, while retaining detectable expression in

leaves. Also, when comparing relative leaf GUS activity versus root and shoot activity, the ratios were about the same as with GFP synthesis. Stem to leaf (1.4 fold in stem) and root to leaf (1.8 fold in roots) GUS activity ratios (Jefferson et al., 1987), were similar to the GFP synthesis level ratios (as percent of total protein) at 1.7 fold with stem to leaves and 1.63 fold higher in roots than in leaves. GFP production was shown to be higher in the mid veins of the artificial light grown mGFP5er transgenic, and elevated GFP synthesis in shoots of the field mGFP5er transgenic also proved to be greater than in leaves. This elevated vascular expression also seems to be consistent with the viral CaMV promoter activity, possibly the result of the proposed role of the native gene in replication (Jefferson et al., 1987; Pfeiffer and Hohn, 1983).

Relative leaf GFP synthesis amounts in the mGFP5er transgenic proved to be slightly higher in leaf primordia and capsules than in the lower fully expanded leaves. Although Jefferson et al. (1987) reported that GUS activity with the CaMV promoter was the same or even relatively higher in older fully expanded leaves than in younger ones, others have found CaMV 35S activity to be higher in newer leaves. For example, GUS-transgenic tobacco that were 120 cm tall (similar to the artificial light grown plants in this experiment) had greater CaMV 35S activity (almost 8 fold) in the highest leaf as compared to the lowest (oldest) leaf (Mitsuhara et al., 1996). It has been suggested that the CaMV promoter is more active in the S phase of the cell cycle (Nagata et al., 1987). This may be the reason for increased meristematic tissue seen here, where cell division activity is elevated.

In this study, it was shown that under control of the CaMV 35S promoter, GFP expression levels are similar to those in experiments where the CaMV promoter is used. Specific GFP characteristics resulting in reduced GFP fluorescence were also not evident by the expression patterns. The optimal position for detection and GFP expression patterns should therefore remain constant when using the same promoter barring position and copy number effects. These results should further develop the use of GFP technology as a tool for any study where GFP fluorescence is detected macroscopically.

Acknowledgements

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