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Quantitative GFP fluorescence as an indicator of recombinant protein synthesis in transgenic plants

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Abstract The utility of green fluorescent protein (GFP) for biological research is evident. A fluorescence-based method was developed to quantify GFP levels in transgenic plants and protein extracts. Fluorescence intensity was linear with increasing levels of GFP over a range that encompasses transgene expression in plants by the cauliflower mosaic virus 35S promoter. Standard curves were used to estimate GFP concentration in planta and in protein extracts. These values were consistent with ELISA measurements of GFP in protein extracts from transgenic plants, indicating that the technique is a reliable measure of recombinant GFP expression. The levels of in planta GFP expression in both homozygous and hemizygous plants was then estimated. Homozygous transgenic plants expressed twice the amount of GFP than hemizygous plants, suggesting additive transgene expression. This methodology may be useful to simplify the characterization of transgene expression in plants.

Keywords Fluorescence · GFP · Protein quantification · Plant transformation · ELISA

Abbreviations *ELISA:* Enzyme-linked immunosorbent assay · *HRP:* Horseradish peroxidase · *GFP:* Green fluorescent protein

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Introduction

Since the first report of its expression in heterologous systems (Chalfie et al. 1994; Neidz et al. 1995), GFP has grown in popularity as a reporter gene in biology and biotechnology research. Its characteristic fluorescence makes it useful for a variety of studies ranging from the regulation of chimeric gene cassettes (Barz et al. 2002; Manak et al. 2002; Wippersteg et al. 2002) to subcellular localization of fusion proteins (Morin et al. 2001; Nagai et al. 2001; Palma et al. 2002) to whole organism expression of transgenes (Hadjantonakis et al. 1998; Gong et al. 2001; Halfhill et al. 2001). The potential of GFP appears to be greater than its current applications as researchers seeking to characterize GFP under novel conditions reveal new uses for the protein (Stewart 2001). This increased potential is especially pertinent to plants, since it is often desirable to quantify gene expression on the plant and in the field.

The purpose of the investigation reported here was to develop a fluorescence-based protein quantification system that would be an improvement over current measurement methodologies. It has been reported that when GFP is purified, fluorescence intensity is directly proportional to protein quantity (Remans et al. 2000). This observation opens the possibility that a standard curve-based methodology may provide an estimation tool. Other studies have noted that brighter plants and tissues contain higher levels of GFP. Harper et al. (1999) compared densiometric GFP estimates from Western blots to raw (unstandardized) levels of fluorescence intensity at 509 nm. Blumenthal et al. (1999) reported that extracts from brighter tissues (measured by spectrofluorometry) resulted in higher quantities of extracted GFP. Neither study coupled these data with a method that would allow for highly predictive and discrete quantification of protein in vivo. For our project, GFP fluorescence was compared to GFP quantity in order to determine if fluorescence-based quantification was possible. These data were then used to convert fluorescence values to estimates of GFP expression using standard

curves. Fluorescence-based estimates from intact plants and protein extracts were compared to GFP ELISA measurements to determine if this methodology provided a reliable and accurate measurement of the recombinant protein produced in transgenic plants.

The resulting methodology was then used to estimate the in planta expression levels of GFP in both homozygous and hemizygous transgenic plants. Halfhill et al. (2003) recently demonstrated that homozygous individuals exhibit greater fluorescence than hemizygous individuals. The hypothesis tested was that two copies of the transgene at a single locus result in double the level of expression of the transgene due to additive transgene expression. If that is the case, then GFP quantification analysis should indicate twice the amount of GFP per mass of plant tissue in the homozygotes compared with hemizygous plants.

Materials and methods

FluoroMax-2 spectrofluorometer

The FluoroMax-2 (Jobin Yvon and Glen Spectra, Edison, N.J.) is a lab-based spectrofluorometer system that utilizes computer data processing (Datamax Spectroscopy Software, Galactic Industries, Salem, N.H.). Excitation light from a 150-W xenon lamp is delivered to the sample through a bifurcated fiber optic cable. For this application, 385 nm was used as the excitation wavelength. A 4-mm rubber protector surrounds the external end of the cable to prevent signal disruption. When scanning, the cable is then directed to 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 (Millwood et al. 2003). This fluorescence signal is then amplified and displayed on a computer monitor in units of counts per second (cps). The fiber optic probe can be placed on plant leaves or above liquid samples.

GFP standard curves

His-tagged GFP, which was purified using standard nickel-affinity chromatography (Gonzalez and Ward 2000), was used to generate standard curves for fluorescence intensity and protein quantity. The GFP was suspended in either deionized water or canola protein extract (*Brassica napus* cv. Westar) to evaluate the effect of varying conditions on fluorescence. A known quantity of GFP was added to 10 μl of deionized water and an equal quantity added to 10 μl of a protein extract from wild-type canola. Soluble protein was extracted in an alkaline solution and kept on ice to limit protease activity (Halfhill et al. 2001). The protein concentration of the extract, 0.7 μg of soluble protein per 1.0 μl of extract, was determined using a Bradford assay. A third condition involved diluting the protein extract by half—to 0.35 μg of soluble protein per 1.0 μl of extract—and generating a third standard curve. The known GFP quantities ranged from 1.0 ng to 1.0 μg. The FluoroMax-2 spectrofluorometer was used to scan each sample from 440 nm to 520 nm during excitation at 385 nm on a black, fluorescently neutral background surface. A fourth treatment included 10 μl of the GFP dilutions suspended in deionized water that were scanned on a wild-type canola leaf surface. The purpose was to determine if the spectral properties of the leaf affected the fluorescence signal of GFP. The fluorescence value at 450 nm, a value outside the GFP fluorescence peak, was used to standardize the scans to minimize the effect of baseline variation (Millwood et al. 2003). The fluorescence intensity of GFP was measured at 508 nm. The values were normalized by subtracting the 508 nm values of the control samples, deionized water or canola extract not containing GFP (Fig. 1). Regression analysis was used to evaluate the association between the fluorescence intensity and GFP quantity. The experiment was replicated four times for each condition.

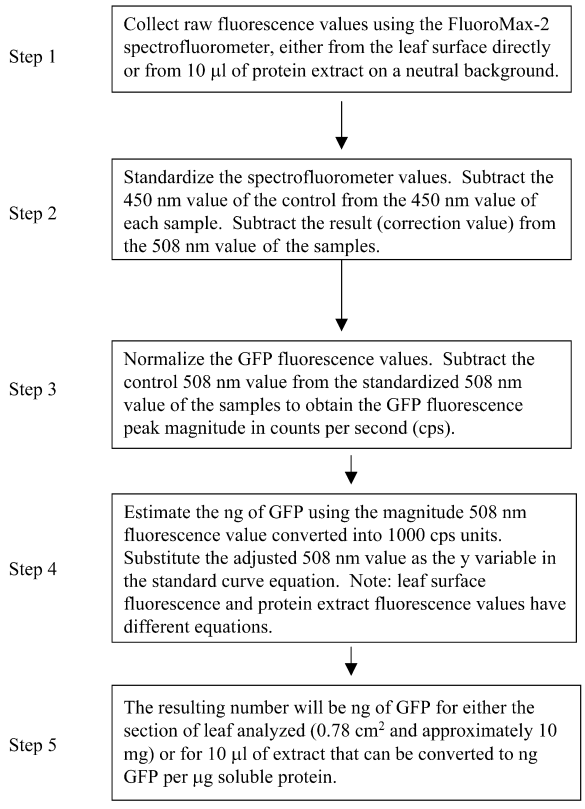

Fig. 1 Flow chart for fluorescence-based quantification of GFP

GFP quantification

Transgenic canola plants were grown under growth chamber conditions at temperatures between 18°C and 22°C under an 8/16-h (light/dark) photoperiod. At the eight-leaf stage (3 months post-germination), spectrofluorescence measurements were taken on the first leaf (youngest), third leaf, and fifth leaf from the apical meristem. The measurement was recorded at the longitudinal midpoint of each leaf, slightly off the midvein. A total of eight transgenic plants were sampled and one nontransgenic control for a total of 27 fluorescence measurements. The transgenic plants were the T_1 generation from a single T_0 line (named GFP1). This line was selected because it exhibited the highest GFP expression in planta of the phenotypically normal events (Halfhill et al. 2001). The fluorescence values were standardized and normalized (Fig. 1), and then the standard curve from leaf surface background used to quantify GFP (y=11.0x−220).

For each of the leaves that were analyzed for fluorescence, 200 mg of tissue were collected for protein extraction. The collected tissue included the area of fluorescence measurement. Soluble protein was extracted (Halfhill et al. 2001) and quantified using Bradford total protein assays. A sample of the extract (10 μl) was analyzed for fluorescence and the values standardized at 450 nm and normalized at 508 nm. GFP was quantified using the standard curve equation from the plant extract controls (y=7.7x−48.3). These values were then compared to ELISA estimates from the same samples.

A sandwich ELISA for GFP was conducted on 10 μl of each leaf protein extract. Samples were incubated for 1 h on Reacti-Bind Anti-GFP plates (Pierce Chemical) coated with polyclonal GFP goat antibodies. Each sample was repeated in triplicate for each plate. Rabbit anti-GFP polyclonal antibody (Molecular Probes, Eugene, Ore.) was added to each well at a 1:10,000 dilution and incubated for 30 min at room temperature. Goat polyclonal antibodies against rabbit IgG conjugated to the HRP enzyme (Sigma, St. Louis, Mo.) were added to each well at a 1:10,000 dilution and incubated 30 min at room temperature. To each well, 100 μl of HRP substrate (Pierce Chemical) was added. After 5 min, 100 μl of 1.0 *N* HCl was added to stop the reaction. The absorbance at 450 nm was measured for each well using a 96-well plate reader. A standard curve was calculated from the averaged values of the standards and used to estimate the amount of GFP in the canola extract samples. The estimated GFP values from both the fluorescence-based and ELISA methods were reported as nanograms GFP per microgram total soluble protein. The values for both methodologies were reported as micrograms GFP per milligram soluble protein and regression analysis performed to determine the association.

Additive transgene expression evaluation

GFP transgenic canola plants (GFP1) were grown under growth chamber conditions. Each plant was of the T_1 generation of a single transgenic event (Halfhill et al. 2001). The zygosity of each plant was determined by progeny analysis of the T_2 seeds. Plants were classified as homozygous if all T_2 seedlings were GFP-fluorescent and hemizygous if any non-fluorescent seedlings were recorded. Twenty seedlings were screened per individual. At the eight-leaf stage (3 months post-germination), spectrofluorometer measurements were taken on three leaf positions on each T_1 plant (youngest, middle, and oldest leaf). These measurements were standardized and normalized as above (Fig. 1) and the resulting values used to estimate the amount of GFP per area of intact leaf tissue based on the leaf surface standard curve (y=11.0x−220).

Results

GFP standard curves

The amount of GFP protein was directly proportional to the fluorescence intensity (Fig. 2). Regression analysis confirmed a strong linear correlation in all assay conditions (R^2>0.99 for all treatments). The plant protein extract reduced the fluorescence intensity of the GFP at each quantity of protein, and the protein concentration had an impact on fluorescence intensity. The higher the concentration of soluble protein, the lower the fluorescence value at a specific quantity of protein. The purified protein suspended in deionized water exhibited a linear relationship from 1.0 ng to 1.0 μg GFP; however, the protein extracts had a lower threshold detection limit at about 25 ng. The fluorescence values taken on the black, fluorescently neutral background were not significantly different from those taken on the leaf surface (P<0.001).

GFP quantification

There was a strong association (R^2=0.90) between the ELISA estimates and the fluorescence-based estimates of GFP in the protein extracts (Fig. 3). In addition, the values

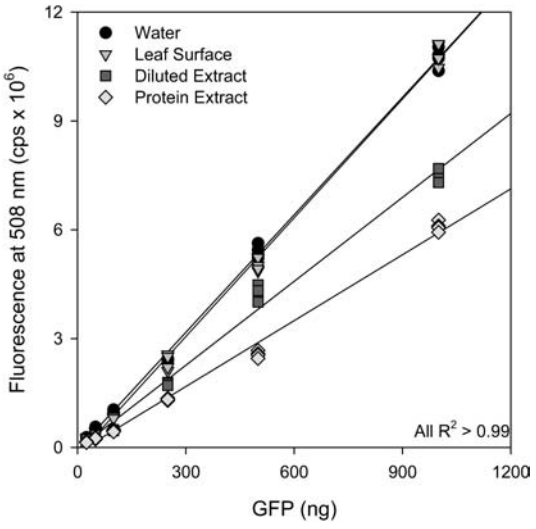

Fig. 2 GFP fluorescence relative to nanogram protein standard curves. Four standard curves were generated by suspending known quantities of purified GFP in either deionized water, canola protein extracts, or on a leaf surface. All of the curves are linear with high regression values, and the regression line equations were used to quantify fluorescent samples with unknown quantities of GFP. At each concentration of GFP, four fluorescence measurements were taken and averaged together for standard curve calculation

of the estimates from both techniques were similar, indicating that the fluorescence-based estimate reliably reported the quantity of GFP in the protein extract. The association between intact leaf fluorescence-based quantification and ELISA estimates extrapolated from the protein extracts was lower (R^2=0.82), but a positive association did exist (Fig. 3). However, there was a large discrepancy (fourfold) between the values of the intact leaf estimates and the extrapolations from the protein extracts using ELSIA.

Additive transgene expression

Homozygous canola from the evaluated transgenic line produced more GFP than hemizygous canola at all three-leaf positions. At the youngest leaf position, homozygous plants produced 6.4±0.17 compared to 2.5±0.15 in hemizygous plants (all data are reported as micrograms GFP/gram leaf tissue). There was a general reduction in the concentration of GFP from youngest to oldest leaf, but homozygous plants consistently demonstrated higher GFP production with 4.4±0.55 and 1.6±0.63 at the middle and oldest leaves, respectively. This is in contrast to hemizygous plants that produced 2.6±1.11 and 1.0±0.28 at the middle and oldest positions. These data indicate that the homozygous plants produced nearly twice the amount of GFP per leaf area than the hemizygous plants at all stages of leaf development.

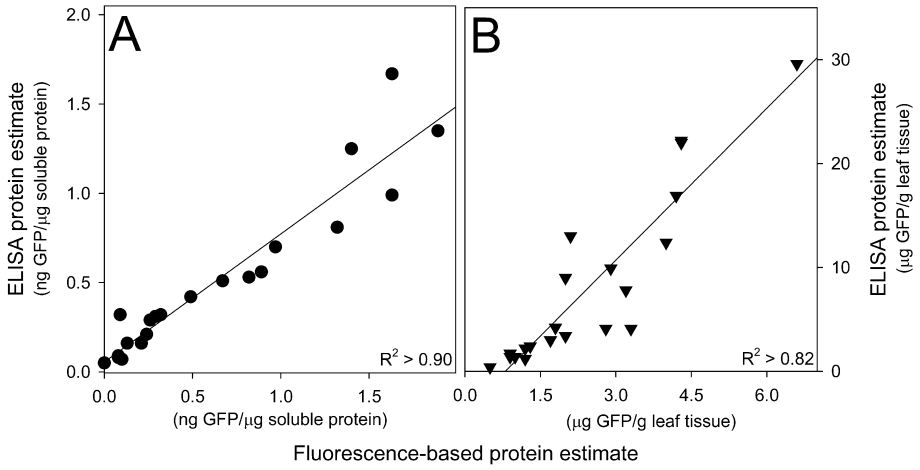

Fig. 3A, B Comparison of GFP concentration estimates from ELISA and fluorescence-based methodology**. A** The GFP content of transgenic canola leaf extracts was estimated using two techniques—ELISA and fluorescence-based quantification. The standard curve from the protein extract in Fig. 2 was used to estimate GFP. There is a strong association between the estimates of the two methodologies. **B** The GFP content of intact leaf material estimated by leaf surface fluorescence using the leaf tissue standard curve from Fig. 2. The protein was extracted from that material and the GFP content estimated by ELISA. There is a strong association between the estimates obtained by both methodologies; however, there is a fourfold discrepancy between the values of the estimates

Discussion

These data indicate that GFP fluorescence is an accurate tool for protein quantification. Fluorescence intensity increases linearly as the quantity of GFP increases; therefore, standard curves can be used to estimate the amount of GFP in an unknown sample. Data from ELISA support the reliability of the fluorescence-based estimates. In measuring GFP in plant protein extracts, both techniques were in agreement, suggesting that the fluorescence-based method is a valid alternative. While there is a strong association between the leaf-surface fluorescence estimates of GFP and the extrapolated estimates from the extracts, there is a discrepancy between the scale of the values. It remains unclear why fluorescence measurements from the leaf surface indicate fourfold less GFP per gram leaf tissue than the ELISA and fluorescence-based estimates from soluble protein extracts.

It is possible that differential expression of GFP in the tissues is a factor. Blumenthal et al. (1999) reported that GFP expression driven by the CaMV 35S promoter in tobacco tissues resulted in heterologous expression in tissue sub-types. This effect was measured by protein blot analysis and fluorescence intensity. A similar effect was observed by Harper and Stewart (2000) and in the transgenic canola line used in this study (Halfhill et al. 2001). The leaf surface estimate measured the fluorescence in a 0.78-cm^2 disk (approximately 10 mg of tissue), while the protein extracts included 200 mg of leaf tissue surrounding that location. This extra material may contain tissue types that have a higher expression (such as vasculature) than the disk measured by the fiber optic probe.

It is also possible that there is interference from plant or fluorescently absorbent compounds that inhibit GFP fluorescence. Remans et al. (2000) also used a fluorescence-based methodology to estimate GFP in transgenic plant extracts. They observed that known quantities of GFP in protein extracts resulted in lower fluorescence than equivalent quantities in the control buffer. This effect was evident when ultraviolet light was used for excitation but was not evident when blue light was used. They hypothesized that compounds in the plant extract may be absorbing the ultraviolet excitation wavelength. In the data presented here, this effect was found to be more pronounced and directly proportional to the density of protein in the extract. If the hypothesis is accurate, then those same compounds may limit GFP excitation in planta. If that is the case, then to bring the leaf surface estimates in line with the extrapolated extract-based estimates, the values could be multiplied by a factor of four.

The three-dimensional structure of the leaf, such as cuticle thickness or cell-wall shape, may also play a roll in the attenuation of measurable GFP fluorescence. Such structural features may refract GFP from excitation or block the emission wavelengths from the detector. These data do indicate that a limitation of GFP fluorescence is not a result of interference from the spectral qualities of the leaf itself (such as autofluorescence of chlorophyll) because purified GFP placed on the leaf surface was not inhibited.

Using this methodology it was possible to determine the difference in recombinant protein expression between the homozygous and hemizygous transgene condition. The homozygotes exhibited twice the level of recombinant protein expression, which supports the additive transgene hypothesis. In these experiments, only one line of transgenic canola was used, and this application must be studied in other lines and species before its effectiveness can be evaluated. However, this methodology may provide a useful tool for transgenic research. Several

questions remain as to how transgene copy number affects expression, and questions pertaining to multiple loci and transgene stacking should play an important role in risk assessment of genetically engineered crops. As the fluorescence-based quantification technique is refined, it may be possible to use GFP-protein fusions to study these questions.

This research extends the utility of fluorescence-base quantification by developing an in planta estimation protocol. Using a FluoroMax-2 spectrophotometer with a fiber optic cable probe, we were able to measure fluorescence directly from the leaf surface, which can then be converted into an estimate of GFP per unit of leaf material. At this time, the probe is 1 cm in diameter, which prohibits tissue-specific estimations, and this methodology will be limited to only fluorescently active molecules. However, it is still an effective tool to rapidly and reliably quantify GFP in planta.

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References

Barz T, Ackermann K, Pyerin W (2002) A positive control for the green fluorescent protein-based one-hybrid system. Anal Biochem 304:117–121

Blumenthal A, Kuznetzova L, Edelbaum O, Raskin V, Levy M, Sela I (1999) Measurement of green fluorescent protein in plants: quantification, correlation to expression, rapid screening, and differential gene expression. Plant Sci 142:93–99

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263:802–805

Gong Z, Ju B, Wan H (2001) Green fluorescent protein (GFP) transgenic fish and their applications. Genetica 111:213–225

Gonzalez DG, Ward WW (2000) Large-scale purification of recombinant green fluorescent protein from *Escherichia coli*. Methods Enzymol 305:212–223

Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A (1998) Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech Dev 76:79–90

Halfhill MD, Richards HA, Mabon SA, Stewart CN Jr (2001) Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa*. Theor Appl Genet 103:659–667

Halfhill MD, Millwood RJ, Weissinger AK, Warwick SI, Stewart CN Jr (2003) Additive transgene expression and genetic introgression in multiple GFP transgenic crop × weed hybrid generations. Theor Appl Genet (in press)

Harper BK, Stewart CN Jr (2000) Patterns of green fluorescent protein in transgenic plants. Plant Mol Biol Rep 18:141a-141i

Harper BK, Mabon SA, Leffel SM, Halfhill MD, Richards HA, Moyer KA, Stewart CN Jr (1999) Green fluorescent protein as a marker for expression of a second gene is transgenic plants. Nat Biotechnol 17:1125–1129

Manak MS, Paul AL, Sehnke PC, Ferl RJ (2002) Remote sensing of gene expression in planta: transgenic plants as monitors of exogenous stress perception in extraterrestrial environments. Life Suppl Biosphys Sci 8:83–91

Millwood RJ, Halfhill MD, Harkins D, Russotti R, Stewart CN Jr (2003) Instrumentation and methodology of GFP quantification in intact plant organs. Biotechniques 34:638–643

Morin X, Daneman R, Zavortink M, Chia W (2001) A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. Proc Natl Acad Sci USA 98:15050–15055

Nagai T, Sawano A, Park ES, Miyawaki A (2001) Circularly permuted green fluorescent proteins engineered to sense Ca^{+2}. Proc Natl Acad Sci USA 98:3197–3202

Neidz RP, Sussman MR, Satterlee JS (1995) Green fluorescent protein: an in vivo reporter of plant gene expression. Plant Cell Rep 14:403–406

Palma E, Mileo EM, Martinez-Torres A, Eusebi F, Miledi R (2002) Some properties of human neuronal alpha 7 nicotinic acetylcholine receptors fused to the green fluorescent protein. Proc Natl Acad Sci USA 99:3950–3955

Remans T, Schenk PM, Manners JM, Grof CPL, Elliott AR (2000) A protocol for the fluorometric quantification of mGFP5-ER and sGFP(S65T) in transgenic plants. Plant Mol Biol Rep 17:385–395

Stewart CN Jr (2001) The utility of green fluorescent protein in transgenic plants. Plant Cell Rep 20:376–382

Wippersteg V, Kapp K, Kunz W, Jackstadt WP, Zahner H, Grevelding CG (2002) HSP70-controlled GFP expression in transiently transformed schistosomes. Mol Biochem Parasitol 120:141–150