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Fluorometric quantification of green fluorescent protein in tobacco leaf extracts

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ABSTRACT

The main use of green fluorescent protein (GFP) is as a reporter system, where the existence of the protein is usually determined visually using fluorescent microscopy. Although fluorescence-based quantification of GFP is possible, background fluorescence in plants and in plant extracts was observed by our group. Another phenomenon we observed that makes quantification difficult is the increased level of GFP fluorescence in *Nicotiana benthamiana* leaf extracts, probably the result of dimerization of GFP molecules promoted by interaction with some component(s) of tobacco extracts. In the current work, the background fluorescence was minimized and the enhancement of GFP fluorescence in tobacco extracts was eliminated with the addition of urea to the measured solution so that a simple quantification assay for the GFP in the tobacco extracts could be established.

Proteins such as green fluorescent protein (GFP),[1] β-glucuronidase (GUS), and luciferase (LUC) are widely used as systems for gene expression studies and as fusion tags to monitor protein localization within the cells, commonly referred to as reporter proteins [1]. Selection of a protein for these applications should be based on its stability under different conditions (e.g., temperature, pH, salinity, denaturant concentration) as well as the reproducibility of its assay. Therefore, to correctly interpret the activity of each reporter protein, it is important to understand their intrinsic properties. Although GUS is a stable enzyme and so is a good choice for the mentioned applications, however its quantification can be affected by the presence of inhibitors, such as sugars and phenolics, in the plant tissues and plant extracts [2,3]. LUC is used mostly for studying the dynamics of in planta gene expression because the newly formed protein is rapidly inactivated [1]. Therefore, these two proteins, GUS and LUC, are only partially suitable for recombinant protein quantification.

These limitations can be largely overcome by GFP, a protein from the jellyfish *Aequorea victoria* that, due to its unique structure, shows a bright green fluorescence when illuminated with ultraviolet (UV) or blue light. GFP is considered to be useful as a reporter protein or a fusion tag because it does not require either substrate or cofactors for its fluorescence, allowing the protein to be detected in vivo [4]. Another advantage is that its fluorescent properties are not hindered by most N- or C-terminal peptide or protein fusions [5,6]. Expression of GFP in plants has been optimized by different approaches, including the removal of cryptic introns that hampered expression of the wild-type sequence [7]. Further modifications at the chromophore region led to variants with shift excitation/emission and fluorescence intensity. The currently known GFP variants can be divided into seven classes based on composition of their chromophores, with each class having a distinct set of excitation and emission wavelengths ranging from 360 to 489 nm for excitation and 440 to 529 nm for emission [4,8]. These GFP versions, used as a reporter genes and fusion tags, have become very important tools in cell biology studies, including subcellular localization of proteins, protease action, transcription factor, dimerization, Ca^{2+} sensitivity, cellular pH alterations, protein and organelle diffusion and movement within the cell, and protein–protein interactions by fluorescence resonance energy transfer (FRET) [4,6,8–10]. In addition, synthetic GFP (sGFP, also referred to as enhanced GFP [EGFP]), GFP172 and GFP157 variants [11], and wild-type GFP [5] were successfully used as a fusion tag for both monitoring and purifying recombinant proteins produced in *Escherichia coli* [11].

One drawback of using GFP in plant systems is the fluorescence from cell wall components, chlorophyll, and other cellular compounds, generally referred as endogenous fluorescence [12–14]. This endogenous fluorescence can interfere in the detection of GFP by fluorescence microscopy, particularly if the total amount of GFP molecules in a given cell is low or if these molecules are not accumulated in a particular organelle [10]. Endogenous fluorescence of plant extracts can also substantially interfere in their quantification [1,15]. The cell wall compounds, chlorophyll, phenolic compounds, NAD, and flavonoids are some of the com-

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[1] *Abbreviations used:* GFP, green fluorescent protein; GUS, β-glucuronidase; LUC, luciferase; UV, ultraviolet; FRET, fluorescence resonance energy transfer; sGFP, synthetic GFP; EGFP, enhanced GFP; BSA, bovine serum albumin; sGFP(S65T), sGFP gene containing a mutation at the chromophore S65T; LB, Luria Broth; IPTG, isopropyl β-D-thiogalactoside; SDS, sodium dodecyl sulfate.

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pounds that can potentially contribute to the fluorescence in plant extracts [1,12,14]. However, because the cell wall compounds are usually not a part of the protein extracts and chlorophyll is only partially soluble in aqueous solutions, the background fluorescence of plant extracts, although present, is much lower than the background fluorescence of plant organs [1]. Yet another potential drawback of using GFP fluorescence to determine its concentration is the increase of its fluorescence as a consequence of the self-association at elevated protein concentrations [16]. These drawbacks—endogenous fluorescence and GFP self-association—are of utmost importance, especially if the GFP fluorescence is used to monitor the fusion protein purification. Because the measured fluorescence will depend on the type of compounds present in the extracts as well as the protein concentration, the error in estimating the GFP concentration could be significant if the above-mentioned drawbacks are not overcome.

In the current work, the limitations of using GFP as a reporter system or fusion tag in tobacco leaf extracts were studied by varying the characteristics of the solution in which the GFP is diluted before quantification (level of dilution, pH, salinity, and denaturant concentration) and by carefully adjusting the fluorometer parameters. Dilution (20×) of tobacco leaf extracts spiked with GFP by the addition of urea (6 mol/L) allowed a correct correlation of GFP fluorescence with its concentration, resulting in the definition of the assay for quantification of GFP in such extracts.

Materials and methods

Materials

Seeds of *Nicotiana benthamiana* and *E. coli* XL1 Blue were provided by the Laboratório de Transferência de Genes (EMBRAPA, Brazil). Bovine serum albumin (BSA, ⩾ 98% electrophoretically pure) and (+)-catechin (⩾ 98% pure) were obtained from Sigma (USA). All other chemicals used were of at least analytical grade. High-purity water prepared with a Milli-Q System (Millipore, USA) was used in all experiments.

Methods

Bacteria expression vector and culture conditions

The sGFP gene, containing a mutation at the chromophore S65T [sGFP(S65T)] [17], was cloned at the *Bam*HI and *Pst*I sites of the bacteria expression vector pQE30 (Qiagen, Germany) to generate a hexa-histidine–GFP gene fusion. *E. coli* strain XL1 Blue was transformed by electroporation and grown on Luria Broth (LB) containing 1.3% agar. Colonies were selected based on GFP fluorescence as observed under a stereomicroscope equipped with a UV lamp and a GFP filter set (excitation 490 nm, barrier filter 515 nm).

Protein quantification

Total soluble protein concentration was determined with the Bradford method [18] using BSA as standard. A DU 650 spectrophotometer (Beckman, USA) was used for absorption measurements.

Production and purification of sGFP

The *E. coli* strain XL1 Blue cells encoding the sGFP(S65T) gene were grown in 0.5 L of LB medium with 100 μg/ml ampicillin at 37 °C after being inoculated with 20 ml of fresh overnight culture. When the culture OD was approximately 0.8, isopropyl β-D-thiogalactoside (IPTG) was added (final concentration of 1 mmol/L) to induce protein expression. Growth was then continued for 2.5 h after induction, and cells were harvested by centrifugation at 5000*g* at 12 °C for 10 min. The cells were sonicated in 20 ml of 20 mmol/L phosphate buffer (pH 7.4) with 300 mmol/L NaCl and 20 mmol/L imidazole (adsorption buffer), and the cell walls were removed by centrifugation at 8000*g* at 4 °C for 15 min. A volume of 10 ml of supernatant was injected into a 5-ml HisTrap column (Amersham Biosciences, Sweden). The column was washed with 100 ml of adsorption buffer. Elution was done with the same buffer containing 200 mmol/L imidazole, and 5-ml fractions were collected. The elution fraction, determined to contain electrophoretically pure (⩾ 98%) sGFP(S65T), was used for spiking tobacco leaf extracts.

Extraction protocol

In each extraction run, 5 g of fresh *N. benthamiana* leaves was macerated in a Waring blender with 50 ml of the extracting solution (50 mmol/L sodium phosphate buffer, pH 7.0) at a 1:10 solid-to-liquid ratio at room temperature for 5 min. Then the suspension was centrifuged at 15,000*g* at 5 °C for 20 min, and the supernatant was polished by filtration through a 3-μm filter paper.

Spiking of tobacco extracts

N. benthamiana leaf extracts spiked with sGFP(S65T) were prepared by adding electrophoretically pure sGFP(S65T) (0.45 mg/ml) to the extracts of fresh leaves. These spiked tobacco leaf extracts contained 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0% sGFP(S65T) (mass of sGFP(S65T): mass of total protein in the extract, $w_{sGFP(S65T)}$) to simulate the in vivo expression of this protein within the tobacco plant.

Fluorometric measurements

Fluorometric measurements were done with an F-4500 fluorescence spectrophotometer (Hitachi, Japan) set to a 480-nm excitation wavelength with an excitation slit opening of 5 nm and a 513-nm emission wavelength with an emission slit opening of 20 nm. One unit of sGFP(S65T) fluorescence was defined as the fluorescence of 1 ng/ml sGFP(S65T) in 50 mmol/L sodium phosphate buffer (pH 7.0).

Results and discussion

To select the GFP variant suitable for application as a reporter system or fusion tag in tobacco extracts, the absorbance of tobacco extracts was first scanned from 300 to 700 nm (Fig. 1). Because the wild-type GFP in its naturally occurring form has a major excitation peak at 395 nm and an emission peak at 508 nm [4], we did not consider it as the most suitable variant for the above-mentioned purposes given that the native tobacco components strongly absorb light at 395 nm and, therefore, would diminish the precision and reproducibility of the fluorescent readings. The enhanced sGFP(S65T) variant with an excitation peak at 480 nm and an emission peak at 513 nm greatly reduces the problem of absorption of light by native compounds (Table 1) [17]. Besides, its fluorescence

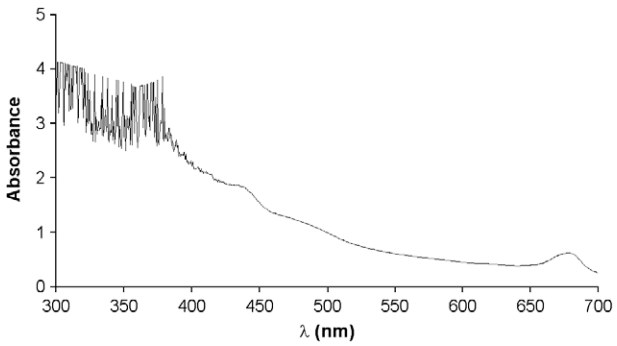

Fig. 1. Absorbance of tobacco leaf extracts obtained with 50 mmol/L sodium phosphate buffer (pH 7.0) at a 1:10 solid-to-liquid ratio at room temperature.

intensity is up to eightfold greater than that of the wild-type GFP [12]. Therefore, it was selected for this work.

Since the work of Remans and coworkers [15] showed that the GFP variant mGFP5-ER (excitation 395 nm and emission 510 nm) has a lower level of fluorescence in tobacco extracts than that in only the extraction buffer, we verified whether a similar phenomenon occurs with the sGFP(S65T) variant. The tobacco extracts were spiked with sGFP(S65T), and the fluorescence of these spiked extracts was compared with the fluorescence of sGFP(S65T) solutions in phosphate buffer with the same sGFP concentrations (Fig. 2). The fluorescence of the sGFP(S65T) in the extracts was found to be approximately twice as high as that in only the extraction buffer. This effect of tobacco extracts on sGFP(S65T) fluorescence is the opposite of that reported previously for mGFP-5ER and shows a different pattern from what was described previously for sGFP(S65T) [15].

The work of Ward and coworkers [16] showed that, by using the wild-type *Aequorea* GFP, the elevated protein concentration leads to self-association of GFP, in turn leading to an increase of its fluorescence. A similar effect of protein concentration on *Aequorea* GFP fluorescence was also reported by Morise and coworkers [19]. Furthermore, by adsorbing aqueorin and GFP on DEAE–Sephadex gel, these authors found that the resin had an effect of approximating the proteins sufficiently close to allow the energy transfer between them. Therefore, we assumed that the enhanced fluorescence of sGFP(S65T) in the tobacco extracts could be the result of dimerization and/or approximation of GFP molecules that could be promoted by the interaction with some compounds present in the tobacco extract. The common reason for the change in protein activity in plant extracts is the association with other proteins as well as the formation of multimers or the interaction with phenolic compounds [20,21].

Therefore, different extraction buffers were used to see whether the interactions responsible for an increase in the sGFP(S65T) fluorescence could be affected. We varied pH and salinity and added reducing agents to the extraction buffer. The increased fluorescence of sGFP(S65T) in tobacco extracts was not affected by pH changes (pH values of 5.0, 6.0, 7.0, 8.0, 9.0, and 12.2) or by the addition of different salts (300 mmol/L NaCl, 10 mmol/L $CaCl_2$, and 2 mmol/L $MgCl_2$) or reducing agents (10 mmol/L β-mercaptoethanol, 10 mmol/L sodium metabisulfite, and 10 mmol/L ascorbic acid).

Next, a set of experiments was run where sugars, a phenolic compound, and a protein were added separately to the extraction buffer containing sGFP(S65T) to determine which of these types of constituent of tobacco extracts might be involved in this fluorescence enhancement. Solutions of sugars (4 mg/ml D-glucose, 3 mg/ml sucrose, and 1 mg/ml dextran), a phenolic compound (0.05 mmol/L (+)-catechin), and a protein (0.4 mg/ml BSA), at concentrations of each class of compound similar to the ones found in tobacco extracts, were prepared, and the fluorescence was measured after the addition of sGFP(S65T) to these solutions.

Table 1
Background fluorescence of tobacco extracts at a 480-nm excitation wavelength with an excitation slit opening of 5 nm and a 513-nm emission wavelength with an emission slit opening of 20 nm.

Dilution of tobacco extracts[a]	Fluorescence units
1	331.5
10	33.2
20	0
100	0
1000	0

[a] The tobacco leaves were extracted with 50 mmol/L sodium phosphate buffer (pH 7.0) at a 1:10 solid-to-liquid ratio and diluted with the same buffer.

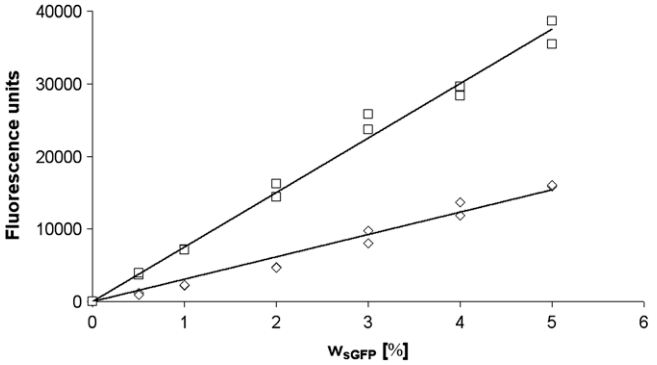

Fig. 2. Fluorescence of tobacco leaf extracts spiked with different concentrations of sGFP (□: slope = 7509, R^2 = 0.99) and sGFP in extraction buffer (◇: slope = 3074, R^2 = 0.98). All of the samples were diluted 20 times in extraction buffer. Extraction conditions: 50 mmol/L sodium phosphate buffer (pH 7.0), 1:10 solid-to-liquid ratio, and 0.40 mg/ml total protein concentration. All of the experiments were done in duplicate and adjusted by the linear fit. w_{sGFP}, protein mass percentage of sGFP in tobacco extracts.

(The solutions of sugars, (+)-catechin, and BSA without the GFP do not have any detectable fluorescence.)

Sugars and phenolics did not produce any detectable alteration on intensity of sGFP(S65T) fluorescence. However, the addition of BSA had substantially increased sGFP(S65T) fluorescence (Fig. 3), suggesting an interaction (aggregation and/or dimerization) of GFP with BSA molecules. Because BSA is not a fluorescent protein, this fluorescence increase could likely result from the approximation of sGFP(S65T) molecules interacting with BSA.

Therefore, we concluded that the interaction of sGFP(S65T) with proteins or some other compound present in *N. benthamiana* leaf extracts is probably the cause of the increase in its fluorescence and that this interaction should be hindered to make GFP quantification based on its fluorescence viable.

The work of Yang and coworkers [22] showed that the driving force of the *Aequorea* GFP dimer formation is the result of both hydrophobic and hydrophylic interactions, the main interactions present in protein aggregation in general [23]. Therefore, a simple and effective approach to hinder the interactions of sGFP(S65T) with other proteins as well as the sGFP(S65T) dimer formation would be to explore the well-known tolerance of GFP to denaturant agents such as guanidine hydrochloride, urea, and sodium dodecyl sulfate (SDS) [24]. The experiments with sGFP(S65T) in different concentrations of urea (as high as 6 mol/L) showed no

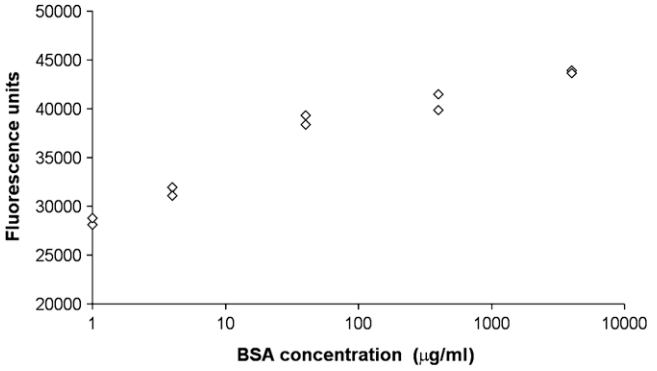

Fig. 3. Fluorescence of sGFP in extraction buffer (50 mmol/L sodium phosphate buffer, pH 7.0) diluted 20 times with different concentrations of BSA in the extraction buffer. (There was no detectable fluorescence readings of BSA solutions used in these experiments.) The solutions of sGFP contained 28,000 fluorescence units before the addition of BSA. All of the experiments were done in duplicate.

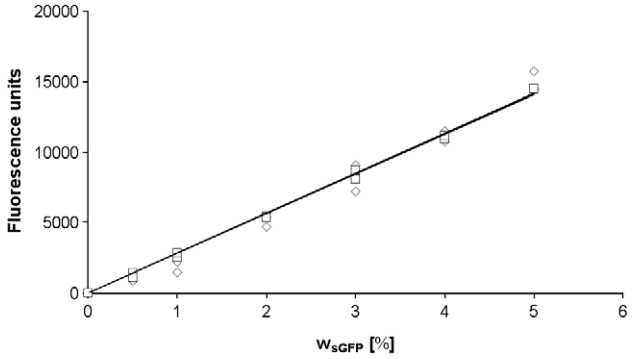

Fig. 4. Fluorescence of tobacco leaf extracts spiked with sGFP at different concentrations (◇: slope = 2819, $R^2 = 0.99$) and sGFP in extraction buffer (□: slope = 2831, $R^2 = 0.98$). All of the samples were diluted 20 times in extraction buffer with 6 mol/L urea. Extraction conditions: 50 mmol/L sodium phosphate buffer (pH 7.0), 1:10 solid-to-liquid ratio, and 0.37 mg/ml total protein concentration. All of the experiments were done in duplicate and adjusted by the linear fit. w_{sGFP}, protein mass percentage of sGFP in tobacco extracts.

effect on its fluorescence. However, a relatively low concentration of SDS (0.25%) caused a total loss of its fluorescence. The addition of urea at high concentrations, therefore, could denature the native tobacco proteins without denaturating the sGFP(S65T) and, as a result, the fluorescence increase effect would be annulled.

A new set of experiments was run where the fluorescence of tobacco extracts spiked with sGFP(S65T) and the fluorescence of sGFP(S65T) in extraction buffer diluted 20 times in 6 mol/L urea solution were compared (Fig. 4). The dilution with urea solution completely eliminated the enhancement of sGFP(S65T) fluorescence in the tobacco extract given that these levels of fluorescence were the same as the levels of pure sGFP(S65T) diluted in the same solution. This could be not only the consequence of the above-discussed denaturation of the proteins that form complexes with the sGFP(S65T) but also the well-known effect of urea on proteins in solutions, which is to prevent the protein aggregation [23] by breaking the intermolecular hydrophilic and hydrophobic bonds [25] and, therefore, also preventing the GFP dimer formation.

Conclusions

Diluting the tobacco extracts spiked with sGFP(S65T) by 20 times with 6 mol/L urea solution in 50 mmol/L sodium phosphate buffer (pH 7.0) completely eliminated the interference of the tobacco extract components on the sGFP(S65T) fluorescence. Therefore, this specific dilution makes quantification of sGFP(S65T) based on its intrinsic fluorescence relatively inexpensive, fast, and reliable. Although this method for quantification of sGFP(S65T) was developed using tobacco leaf extracts, it can probably be applied to other plant extracts or other protein solutions as well. The quantification method described may also be applicable to the other variants of GFP so long as the solution in which the GFP will be measured does not absorb the light at the excitation or emission wavelength of the GFP variant used.

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